

IN VITRO ACTIVITY OF SORGHUM NON-TANNIN POLYPHENOLS  
ON GROWTH OF POTENTIAL MYCOTOXIN-PRODUCING FUNGI

by

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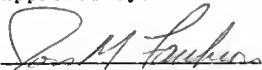
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## INTRODUCTION

Sorghum is the common name given to Sorghum bicolor (L.) Moench. It is the third leading cereal crop produced in the United States and ranks fifth behind rice, wheat, corn, and barley in total world production (Hahn et al 1984). Sorghum is used primarily for feed in the United States. However, it is a staple food in many African countries and some countries of Asia, Central America and the Middle East. Approximately 300 million people rely on sorghum for their sustenance. A potential export market, therefore, exists for sorghum produced in the United States. The U.S. is the leading country in the production of sorghum and also is the principal sorghum exporting country. Export trade, however, demands that sorghum grain must meet a variety of criteria not encountered in domestic sales for feed. These criteria include color, good millability, and freedom from kernel deterioration.

Throughout the world, a barrier to the production of sorghum grain of ideal quality is grain deterioration, caused largely by fungal invasion of the kernel. Deterioration has a number of negative consequences for the grain (Glueck et al 1978). Yield is diminished. The processing properties, handling characteristics and storage properties are altered, and the grains' acceptability as food is reduced severely.

It is evident that sorghum grain from some (but not all) cultivars is less severely damaged by fungi than others. Therefore, sorghum varieties vary in their ability to resist fungal invasion. The resistance mechanism is not understood but it is known that high-tannin sorghum varieties are often relatively more

resistant to fungi, bacteria, and viruses (Friend 1977, and Kosuge 1969), birds (Bullard and Elias 1980, and Butler 1982), and insects (Woodhead et al 1980). However, since tannins produce astringent flavor and reduce the grain's nutritional value (Bullard and Elias 1980, Butler 1982, and Price and Butler 1977, 1980), they are not generally acceptable for food use. The non-tannin polyphenolic compounds in other plant systems are known to function as antifungal agents (Baranowski et al 1980, Byrde et al 1960, Friend 1977, and Pickman et al 1984). Since sorghum is known to contain a variety of these compounds (Hahn et al 1983), they may be active in providing at least part of the resistance to fungal invasion.

The ideal sorghum varieties for the sorghum producer and consumer are those that are low in tannin content, high in nutritional value and with some resistance to pests. Such varieties need to be identified and tested under lab conditions. This is particularly true for assessing levels of resistance to fungal pests. Simple methods for extraction of potentially active compounds and an in vitro analysis method for the extracts could make it possible to analyze the effects of sorghum non-tannin polyphenols on fungal growth without relying on inconsistent field molding situations. Studies utilizing such methods may increase our understanding of the relationship between endogeneous non-tannin polyphenolic compounds and resistance of the grain to fungal attack. This could, in turn, contribute to the development of resistant varieties retaining good end use properties.

The objectives of this research were: 1) to extract kernel polyphenols from two differing sorghum cultivars and test them in vitro for activity against grain molding and weathering fungi,

especially potential mycotoxin-producing fungi; 2) to determine the effects of standardized concentrations of phenolic acids on fungal growth and 3) to separate and quantify the phenolic acids of the above mentioned sorghum varieties by high performance liquid chromatography.

## LITERATURE REVIEW

### Phenolic compounds

All sorghums contain phenolic compounds which can affect the color, appearance, and nutritional quality of the grain and resultant sorghum products (Hahn et al 1984). The phenolic compounds can be divided into three broad groups: phenolic acids, flavonoids, and condensed tannins. All sorghums contain phenolic acids and all those so far studied contain flavonoids (Hahn et al 1984). The term "phenolic acid" is applicable to a large variety of different organic compounds bearing at least one phenolic hydroxyl group and a carboxyl function (Stumpf and Conn 1981). As a group, phenolic acids comprise the benzoic and cinnamic acids as well as their derivatives (Ribereau-Gayon 1972). Benzoic acids (phenylcarboxylic acids) have a seven carbon  $C_6-C_1$  skeleton (Fig. 1B) and cinnamic acids (phenylacrylic acids) possess nine carbon  $C_6-C_3$  skeleton (Fig. 1A). Their derivatives are hydroxylated and/or methoxylated at various sites on the aromatic ring nucleus.

The carbon skeleton of cinnamic acids originates from L-phenylalanine (Stumpf and Conn 1981). The primary pathway for the biosynthesis of benzoic acids in plants is the side-chain degradation of cinnamic acids (Stumpf and Conn 1981).

Benzoic acids are widely distributed in plants, the most common being p-hydroxybenzoic, protocatechuic, vanillic, gallic, syringic, salicylic, and gentisic acids (Ibrahim and Towers 1960). Four of the cinnamic acids are known to be widely distributed in the plant kingdom: p-coumaric, caffeic, ferulic, and sinapic acids (Ibrahim and Towers 1960, and Ribereau-Gayon 1972). In nature

Fig. 1. Schematic structures of phenolic compounds.

A. cinnamic acid.

B. benzoic acid.

C. flavonoid.

D. proanthrocyanidin (tannin)  
polymer; (n=2-4).

(Figures from Hahn et al 1984).

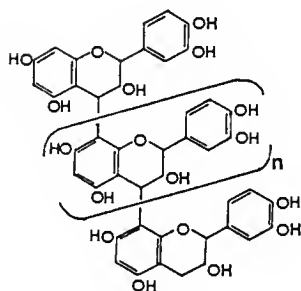
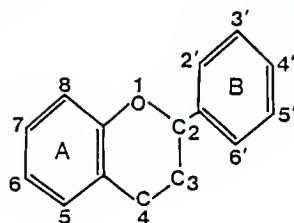
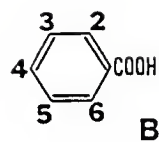
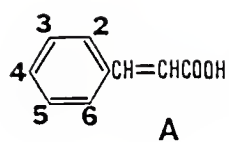
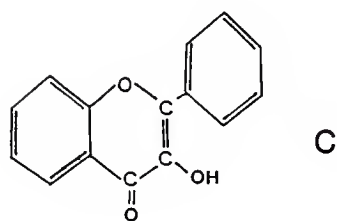
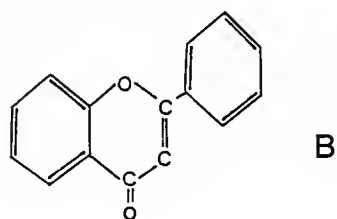
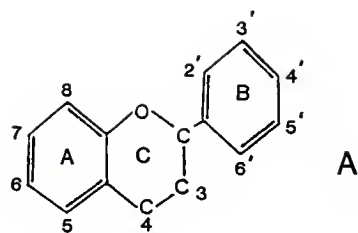


Fig. 2. Structurally distinct groups of flavonoids nuclei.

A. flavans.

B. flavones

C. flavonols.



cinnamic acids occur as the more stable trans isomers. When exposed to light, the isomers are easily interconvertible and reach an equilibrium condition. It is clear that the equilibrium is affected by the compound itself, its molar concentration and the pH of the solvent (Kahnt 1967). The phenolic acids most often occur either as esters of sugars or glycosides (Pridham 1960, Towers 1964, and Kosuge 1969) or as free acids (Ibrahim and Towers 1960, Krygier et al 1982a, and Wulf and Nagel 1976). Wulf and Nagel (1976) identified three classes of naturally occurring phenolic compounds: phenolic acids (both benzoic and cinnamic) and a variety of flavone compounds including flavones, flavonols, and flavanones as well as glycosylated flavone derivatives with mono- and disaccharide substitution. Krygier et al (1982a) and Soluski et al (1982) devised a fractionation system to separate the phenolic constituents of cereal, potato, and rapeseed flours into free, esterified, and insoluble-bound phenolic acids.

The second group, the flavonoids, are the largest group of phenolic compounds in the plant kingdom with almost two thousand different flavonoids now identified (Harborne et al 1975). Structurally the flavonoids are characterized by their fifteen carbon skeleton ( $C_6-C_3-C_6$ ) (Fig. 1C). They consist of two distinct units: A  $C_6-C_3$  fragment from cinnamic acid forms the B-ring, and a  $C_6$  fragment derived from malonyl-CoA forms the A-ring.

Flavonoids are divided into three structurally distinct groups: flavones, flavonols, and flavans. Flavones (Fig. 2B) possess a carbonyl at  $C_4$ , and a double bond between  $C_2$  and  $C_3$ ). Flavonols (Fig. 2C) contain a carbonyl at  $C_4$ , and hydroxyl at  $C_3$ . Flavans (Fig. 2A) resemble flavones but lack the carbonyl at  $C_4$ . The predo

minant flavonoids in sorghum are the flavans (Hahn et al 1984). The major flavans are leucoanthocyanidins (Flavan-3,4-diols possessing hydroxyls at  $C_3$  and  $C_4$ ), catechin or 4-deoxyleucoanthocyanidins (Flavan-3-ols with hydroxyls at  $C_3$ ), and anthocyanidins (Flavan-3-en-3-ols possessing a hydroxyl at  $C_3$ , and double bond between  $C_3$  and  $C_4$ ). Anthocyanidins are defined as being capable of generating anthocyanin. Flavonoids generally occur as glycosides (Wulf and Nagel 1976). The flavonoid nucleus without its saccharide is termed an aglycone.

A number of flavonoid compounds are known to exist in the mature seed of sorghum (Butler 1982, Hahn et al 1984, Nip and Burns 1968, and Olifson et al 1971). The pericarp color of sorghum appears to be due to a combination of anthocyanin and anthocyanidin pigments and, to a lesser, extent other flavonoid compounds.

There appears to be a good deal of variation in flavonoid composition between sorghums of the same genetic pericarp color (Hahn et al 1984). Flavonoid compounds are known to be present in the pericarp of both white and red sorghum varieties (Kambal and Bate-Smith 1976, Nip and Burns 1969 and 1971).

Tannins are a group of phenolic compounds found in many plants. The term "tannin" is defined as a high molecular weight polymeric phenol with the ability to combine with protein and other polymers such as cellulose and pectin (Leungchaikul 1982). Tannins can be characterized as being hydrolyzable or condensed. Hydrolyzable tannins are tannins that are readily hydrolyzed either chemically or enzymatically. Hydrolyzable tannins often termed vegetable tannins (eg. tannic acid) break down into sugars and a phenolic acid (eg. gallic acid or ellagic acid) when treated with acid,

alkali, or some hydrolytic enzymes (tannase) (Gupta and Haslam 1980, and Hahn et al 1984). Only condensed tannins, polymers resulting from the condensation of hydroxyflavans (flavan-3-ols or catechins and flavan-3,4-diols or leucoanthocyanidins), have been found in sorghum. These polymers are now referred to as proanthocyanidins (Fig. 1D), since anthocyanidins are released when the tannins are treated with mineral acids. Gupta and Haslam (1978) refer to sorghum tannins as procyanidins, due to their contention that cyanidin, flavan-3-ols: (+)-catechin or (-)-epicatechin, is usually the sole anthocyanidin involved.

#### Sorghum polyphenols

In cereal grains such as sorghum, the phenolic compounds are concentrated in the outer layers of the kernel (pericarp, testa, and aleurone). Only insoluble, tightly bound phenolic acids are found in the endosperm in low amounts (Hahn et al 1984). The bound or insoluble phenolic acid esters appear to be associated with the cell walls of the grain (Hahn et al 1984). The major bound phenolic acid of sorghum is ferulic acid (3-methoxy-4-hydroxy-cinnamic acid), which is thought to be associated with the cell walls (Hahn et al 1983, and Rooney and Miller 1982). Condensed tannins are found in the testa layer (Blakely et al 1979) and, depending on the genetics of the seed, the mesocarp. Precise cellular locations as well as the distribution of specific non-tannin polyphenolic compounds (particularly phenolic acids) in the sorghum kernel are not known with certainty.

## Genetics

Sorghum classification based on chemical analyses and structural features was proposed by Cummings and Axtell (1973) and redefined by Price and Butler (1977). The chemical criteria involve phenolic compounds. Using various characteristics, three groups are defined. Group I sorghums lack a pigmented testa. Sorghums classed as group II have a pigmented testa but lack a dominant spreader gene. Group III grain has a testa and dominant spreader gene (Rooney and Miller 1982). The presence of a pigmented testa is controlled by the complimentary  $B_1$  and  $B_2$  genes. Both must be dominant ( $B_1-B_2-$ ) before a pigmented testa can be present. The spreader gene ( $S_-$ ) controls the spread of the phenolic pigments and possibly tannins into the epicarp. When S is dominant, more phenols and tannins exist in the pericarp and testa layers. Type III sorghums have the greatest bird resistance and, interestingly, the highest level of tannins (Rooney et al 1980, and Rooney and Miller 1982). Their presence results in type III grain having a reduction in nutritional value relative to types I & II (Rooney et al 1980, and Rooney and Miller 1982). It has been suggested that the type II brown sorghums contain tannins during maturation (which provide bird resistance) and that the tannins disappear upon maturation (Hahn et al 1984). In this way, they do not adversely affect the nutritional value of the grain.

## Plant phenolics and disease resistance

The most common causes of plant diseases are fungi, bacteria, and viruses (Agrios 1969). In many cases, the compounds responsible for disease resistance in nature are phenolics or polyphenols

(Friend 1977). Brown sorghums, with high tannin contents, are relatively more resistant to molding and weathering than nonbrown sorghums (Bullard and Elias 1980, and Hahn et al 1984). However, tannins which produce astringent flavor and, thereby, repellency also reduce the palatability, digestibility, and nutritional quality of many foods containing them (Bullard and Elias 1980, Butler 1982, Chang and Fuller 1964, Cumming and Axtel 1973, and Price and Butler 1977, and 1980). This is due to the binding and precipitation of proteins by condensed tannins (Gupta and Haslam 1980). Some nonbrown or low-tannin sorghums, which are low in tannin content, do have appreciable resistance to molding and weathering (Hahn et al 1983), which suggested that these varieties may have polyphenolic compounds important in fungal resistance. In low-tannin sorghums, many of these compounds are likely to be phenolic acids.

Many phenolic acids have shown antifungal activities in other plant systems (Baranowski et al 1980, Byrde et al 1960, Friend 1977, and Pickman et al 1984). Pickman et al (1984) reported that phenolics and some other, unidentified, compounds are chemical antifungal agents in oat hulls. Catechol and protocatechuic acid in onion appear to act as inhibitors of spore germination of the fungus Colletotrichum circinans (Friend 1977). Ferulic, p-coumaric, vanillic, and p-hydroxybenzoic acids inhibit the growth of Saccharomyces cerevisiae (Baranowski et al 1980). Hydroxycinnamate derivatives were found to be inhibitory to Trichophyton rubrum, Aspergillus niger, and Saccharomyces cerevisiae (Gupta and Banerjee 1976). Leungchaikul (1982) found that purified ferulic, vanillic, and p-coumaric acids inhibit the growth of common sorghum grain

weathering fungi.

Mold growth occurs in two stages: germination of the spores followed by mycelial growth. Specific plant phenolics are known to inhibit both stages (Friend 1977). In addition to direct inhibition of fungal growth, protective activity can be conferred by the oxidation of pre-existing phenolic compounds (Byrde et al 1960, and Walker 1969). Such protective activity can also be caused by the inhibition of fungal extracellular enzymes, inhibiting cell-wall degradation and penetration by the mycelia (Hunter 1974 and Leungchaikul 1982).

#### Extraction and separation of phenolic acids of sorghum grain

Several methods have been used to isolate cereal polyphenols. The most common extraction solvents are water (Blessin et al 1963, and Price and Butler 1977), acetone (Kaluza et al 1980, and Pickman et al 1984), ethanol (Butler et al 1980, Gupta and Haslam 1980, Kaluza et al 1980, and Strumeyer and Malin 1975), methanol (Burns 1971, Hagerman and Nicholson 1982, Leungchaikul 1982, and Yasumatsu et al 1965) or acidified methanol (Maxson and Rooney 1972, Nip and Burns 1968). Mixed solvents of methanol, acetone, and water are also employed as extraction solvents (Krygier et al 1982a). The conditions of extraction have been quite varied.

Several methods have been used for qualitative and quantitative analysis of plant phenolics. Paper (Ibrahim and Towers 1960) or thin-layer (TLC) chromatography (Newby et al 1980, and Vande Castele et al 1981) has been used extensively to separate phenolics. It is, however, laborious and difficult to quantitate. More modern procedures involve the use of gas-liquid chromatography

(GLC), gas chromatography-mass spectrometry (GLC-MS) (Krygier et al 1982a&b, Sosulski et al 1982, and Vande Castelee et al 1981) and high performance liquid chromatography (HPLC) (Hagerman and Nicholson 1982, Hahn et al 1983, Murphy and Stutte 1978, and Wulf and Nagel 1976).

### Sorghum fungi

Seed deterioration is caused primarily by fungal invasion of the kernel. Fungi affect sorghum seed quality in many ways. Based on their behavior in attacking the kernel, there are two types of fungi: field and storage fungi (Christensen and Sauer 1982). Field fungi may be further divided into grain molding and grain weathering types. Grain molding is a pre-maturity infestation mainly caused by parasitic field fungi such as Curvularia lunata and Fusarium moniliforme (Castor 1981). Grain weathering is post-maturity field infestation caused by saprophytic field fungi, primarily Alternaria spp. The difference between grain molding and grain weathering is not perfectly distinct. The fungi that cause grain molding can also be involved in grain weathering. As opposed to damage by the storage fungi, the damage done by field fungi is caused before harvest and usually does not increase during storage. Storage fungi can invade kernels prior to harvest, but the greatest invasion of kernels occurs after harvest. Storage fungi generally are members of the species Aspergillus and Penicillium (Castor and Frederikson 1982). Generally, sorghum samples with a high incidence of storage fungi have a very low incidence of field fungi and vice versa (Niles 1976, Pettit and Taber 1978, and Seitz et al 1975).

Dozens of fungal genera occur naturally in sorghum grain (Swarup et al 1962, and Williams and Roa 1980). The most common fungi are members of the fungal genera Fusarium and Alternaria, especially F. moniliforme, F. semitectum, and A. alternata. Other genera found in weathered grain are Helminthosporium, Curvularia, Phoma, Rhizopus, Epicoccum, Olipitrichum, and Aspergillus (Pettit and Taber 1978).

### Fungi in sorghum

Several fungi found in sorghum grain have the potential to produce mycotoxins. The most serious of mycotoxin producers are Aspergillus flavus, F. moniliforme, and Alternaria alternata (Pettit and Taber 1978). A. flavus is seldom found in sorghum grain. However, the incidence of A. flavus in blended feed containing sorghum can be quite high (especially in poultry feed) (Pettit and Taber 1978). A. flavus produces one of the most poisonous classes of substances known, the aflatoxins, a member of a group of mycotoxins which cause liver cancer and many other problems in animals and man. F. moniliforme is capable of producing several mycotoxins, one of the most serious of which is zearalenone. Zearalenone is an estrogenic toxin capable of interfering with the normal reproductive processes in poultry and other farm animals (Pettit and Taber 1978). A. alternata produces a series of toxins: tenuazonic acid, alternariol and its monomethyl ether, alternuene, alternotoxin I (Watson 1984). These compounds have been found together in food samples and may have synergistic toxic effects. Zearalenone, alternariol, and aflatoxins are reported to occur naturally in sorghum grains (Young and Fulcher 1984). Alternaria metabolites

have been primarily associated with grain sorghum (Burroughs et al 1976, Sauer et al 1978, Seitz et al 1975). Metabolite extracts of E. moniliforme and A. tenuissima were highly toxic to day-old cockerels (Diener et al 1981). The presence of aflatoxin and zearalenone was reported in Georgia grain sorghum during maturation (McMillian et al 1983). Other fungi isolated from sorghum also produce mycotoxins, but most of these mycotoxins occurred in lower concentrations. The potential still exists that some of these fungi may be producing potentially serious poisons (Pettit and Taber 1978).

Fungal infection of grain in the field and/or during storage can be a most serious problem. The extent to which grain sorghum is damaged by fungi and the degree to which these grains are contaminated with mycotoxins are dependent on several factors including the climatic conditions under which the grain is produced, the sorghum variety planted, availability of nutrients for plant growth (e.g., calcium), plant stress during the maturation of the grain, and conditions under which the grain is harvested, transported and stored. Since different fungi are capable of growing on the grains, the fungal type which is most active at any time is dependent upon the indigenous fungal population, moisture levels of the grain, temperature, atmospheric humidity, and physiological characteristics of the grain (Pettit and Taber 1978).

#### Location of fungi

Fungi are found more frequently in the pericarp and outer layers of the grain, and fungal numbers gradually decline from outside to inside of the kernel (Mathur et al 1975). Most fungi

(except *E. moniliforme*) do not invade the embryo. *E. moniliforme* is largely present in the inner tissues of the kernel, mostly the endosperm (Mathur et al 1975).

## MATERIALS AND METHODS

### Sorghum samples

Grain of two sorghum cultivars was used for all studies (see Table 1). They were classified as group I, or III using the criteria of Cummings and Axtell (1973). The samples were: "CS3541", a group I sorghum with a thin, white pericarp and intermediate endosperm hardness. "SC0719" was a group III brown-seeded, bird-resistant sorghum with thin pericarp and intermediate endosperm hardness. The grain samples were obtained through the courtesy of Dr. M. M. Morad, Cereal Quality Lab, Texas A&M University, College Station, TX.

All grain samples were air-dried at room temperature to < 11.5% moisture and stored at room temperature in a stainless steel can. Before extraction, they were ground in a Udy laboratory mill (Udy Company, Boulder, CO 80302) to pass a 1.0 mm. screen and stored briefly in the dark at 0°C.

### Growth media

Three media were used to grow fungi:

Malt salt agar + 4% NaCl (MS-4)

Malt salt agar + 4% NaCl + 200 ppm Tergitol (MS-4T)

Potato dextrose agar (PDA).

All media were autoclaved at 121°C (15 psi) for 15 min. (slow exhaust), cooled for 15-30 min., then poured into 100 x 15 mm plastic disposable petri dishes. After the media was left to harden at room temperature, the plates were stored right side up at 4°C overnight or until used.

Table 1  
Sorghum Kernel Characteristics

Sorghum Sample	Pigmented Testa	Pericarp Color	Mesocarp Thickness	Endosperm	Endosperm Texture <sup>a</sup>	Resistance to Fungi <sup>b</sup>	
						Molding	Weathering
CS3541	-	white <sup>c</sup>	thin	white	3	2.5	3.3
SC0719	+	red <sup>d</sup>	thick	white	5	1.5	1.0

a => 1(corneous) to 5(floury) .

b => 1(excellent resistance) to 5(very poor resistance) .

c => or colorless.

d => spreader gene present.

(Ref. from Hahn et al 1983 and Leungchaikul 1982) .

### Fungal isolation, identification, and selection

Fungi commonly associated with weathered or molded sorghum, including potential mycotoxin-producing fungi (Pettit and Taber 1978) were used. The fungi isolated and grain sources are listed in table 2.

Fusarium and Alternaria sp. were isolated from sorghum grains using sterile surface technique. Sorghum kernels were washed 1 minute, in 2% sodium hypochlorite (NaOCl) and rinsed in sterile distilled water to terminate the activity of the chlorine. Twenty five (25) treated sorghum kernels were placed on MS-4T (Seitz et al 1982) with sterile forceps. A total of one hundred and fifty (150) seeds were plated. The plates were incubated at 21-24°C (70-75°F) for 3-5 days. Seeds were observed under a binocular dissecting microscope and appropriate colonies growing from seeds were picked and transferred to PDA. These isolates were grown at 21-24°C (70-75°F) for 5-7 days and the cultures were checked for purity. Pure fungal isolates were, again, picked, grown out, then stored on PDA plates or PDA slants in a refrigerator until use. Isolates of each fungus were selected from colonies grown from different kernels of the same or different varieties of sorghum grown in the same or different plates.

A. flayus was isolated from an infected corn cob onto MS-4. The plates were incubated at 21-24°C (70-75°F) for 48 hours and stored at refrigerator temperature.

Fusarium semitectum and F. equiseti isolates were identified through the courtesy of Dr. Paul E. Nelson, Fusarium Research Center, Pennsylvania State University, State College, PA (Toussoun and Nelson 1976). Alternaria isolates were identified by Dr. Emory

TABLE 2  
Fungal Isolates and Sources

FUNGAL ISOLATES	GRAIN SOURCES
<u>Fusarium moniliforme</u>	Corn (G-4507)
<u>Fusarium semitectum</u>	sorghum
<u>Fusarium equiseti</u>	sorghum
<u>Alternaria alternata</u>	sorghum
<u>Alternaria tenuissima</u> complex	sorghum
<u>Aspergillus flavus</u>	infected corn

G. Simmons, Mycological Services, Amherst, Massachusetts. Fusarium moniliforme was kindly provided by Dr. Barbara A. Hetrick, Department of Plant Pathology, Kansas State University. It was isolated from corn (G 4507-1) using Komada media (Komada 1975). Alternaria alternata isolate (FN 8442-5) (Sauer et al 1978) was supplied through the courtesy of Dr. David B. Sauer, U.S. Grain Marketing Research Laboratory, Manhattan, KS. Three isolates each of F. moniliforme, F. semitectum, F. equiseti, A. alternata, A. tenuissima cpx., and A. flavyus were obtained.

#### Grain extraction

Both free and bound phenolic acid extractions were by the method Hahn et al (1983) (see Fig. 3).

#### Colorimetric quantitation of phenolic compounds

The level of phenolic compounds present in the sorghum samples and all the extracts was determined by two methods; the prussian blue (PB) method (Price and Butler 1977), and the vanillin test (Earp et al 1981).

Materials. Ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , lumps) and potassium ferricyanide ( $\text{K}_3\text{Fe}(\text{CN})_6$ , crystal) were obtained from Mallinckrodt, Inc. (St. Louis, MO), vanillin from Sigma Chemical Company (St. Louis, MO), and D-catechin from United States Biochemical Corporation (Cleveland, OH).

The PB method is reported to measure total polyphenols (Earp et al 1981, Hahn et al 1984, and Price and Butler 1977). A catechin standard curve from 0.0 - 0.24 mg/60 ml was the basis for quantitating polyphenol levels (Leungchaikul 1982). The vanillin

Fig. 3. Free and bound phenolic acid extraction procedure.

Ground Sorghum		
+ 100% MeOH		
(1:4 sorghum to MeOH ratio)		
Extract 30 min., at R.T.		
with Shaking and Centrifuging		
5000 rpm., 30 min. (2X)		
Supernatant (*)	Residue	
Vacuum Evaporate	Hydrolyze 1 hr. with 2 N HCl	
to Near Dryness	(1:4 residue to HCl ratio)	
	in a Boiling Water Bath	
Re-dissolve in	Cool to R.T.	
MeOH (HPLC grade)	Centrifuge	
Free Extract		
HPLC		
In Vitro Testing	Supernatant	Residue
	Extract with EA (1:4 ratio)	
	(2X)	
	Pooled Extracts	
	continue same as (*)	
	Bound Extract	

test was that of Earp et al (1981). It included both the vanillin hydrochloric acid (V-HCl) procedure of Burns (1971), and the modified vanillin hydrochloric acid (MV-HCl) procedure of Maxson and Rooney (1972). The vanillin test measures leucoanthocyanidins (catechins) and proanthocyanidins (tannins), as well as other polyphenolic compounds such as dihydrochalcones and flavanones (Earp et al 1981, Hahn et al 1984, and Sarkar and Howorth 1976). A catechin standard curve from 0.0 - 1.0 mg/ml in 0.2-mg increments was used in calculating polyphenol levels (Earp et al 1981). The results of both methods were transformed to mg/g of sample by the method of Price and Butler (1977).

#### HPLC separation of phenolic acids

Chromatographic separation of phenolic acids was modified from the method of Hahn et al (1983), by the replacement of butanol in solvent B with 2-propanol. Eight phenolic acids were used as standards: gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, ferulic, and cinnamic acids. Gallic, protocatechuic, p-hydroxybenzoic, vanillic, p-coumaric, and ferulic acids were obtained from Sigma Chemical Company (St. Louis, MO), caffeic acid from H.M. Chemical Company (Santa Monica, CA), and cinnamic acid from Fisher Scientific Company (Fair Lawn, N.J.). HPLC grade methanol and 2-propanol from Fisher Scientific Company (Fair Lawn, N.J.) and water from Burdick & Jackson Laboratories, Inc. (Muskegon, MI) were used. All other chemicals were reagent grade.

Instrumentation. A Varian model 5000 high performance liquid chromatograph (Palo Alto, CA) was used in the studies equipped with an Ultrasphere<sup>TM</sup>-ODS column (5  $\mu$ m) of 4.6 mm (i.d.) x 15 cm from

Altex Beckman (Berkeley, CA). The guard column was a 4.6 mm (i.d.) x 5 cm column (pellicular, reverse-phase) from Alltech Associates (Deerfield, IL). Detection was by UV absorption at 254 nm.

**Separation.** To optimize chromatographic separation, grain extracts were dissolved in HPLC grade methanol and filtered through a 0.2- $\mu$ m pore size Swinney-13 Filter Unit (Millipore Corporation, Bedford, MA), then applied to a 1 x 0.8 cm column (C-18 Sep-Pak Cartridge, Water Associates (Milford, MA). High molecular weight polyphenols in the sample were absorbed and retained as a yellow-brown band in the top few millimeters of the column. After applying the extracts, the column was washed with 1.5 ml of methanol (HPLC grade) to elute cinnamic and benzoic acids (Hahn et al 1983). For HPLC, a 10  $\mu$ l sample was injected and eluted at a flow rate of 0.5 ml/min.

Optimum separation of acid standards was obtained by a multi-step gradient of the following solvent mixtures: A, acetic acid-water (2:98); and B, 2-propanol-methanol (8:92). The separation was programmed isocratically for 10 min at 5% solvent B, followed by a 7.5 min linear gradient to 15% solvent B. This intermediate mixture was then programmed isocratically for 13.5 min, followed by a 10-min linear gradient to 50% solvent B. Acetic acid was included to suppress ionization of the carboxyl hydrogens of the benzoic and cinnamic acid nuclei (Hahn et al 1983).

#### Extraction efficiency

Recoveries of the phenolic acid standards by both extraction methods were tested as follows. Ten (10) ml of a 1  $\mu$ g/10  $\mu$ l solution of the standard phenolic acid mixture was processed

through all the steps of each of the extraction methods. As a final step, it was redissolved in 10 ml methanol (HPLC grade) to the final volume. Both non-extracted phenolic acid standards and extracted phenolic acid standards were chromatographed as above. The percent recovery of each phenolic acid standard was calculated as the amount recovered divided by amount added.

#### Preparation of sorghum extracts

Extracts of all sorghum samples were used to test in vitro for their activities on fungal growth. The levels of polyphenols present in each extract were expressed as mg catechin equivalent/ml PDA.

After evaporation to near dryness, the extracts were dissolved in a minimum volume of 80% ethanol. The redissolved extracts were aseptically incorporated in warm, unsolidified PDA after it had been autoclaved. To minimize differences in pH, PDA for in vitro analysis was prepared with 0.2 M phosphate buffer solution, pH 6.5 (Colowick and Kaplan 1955), instead of distilled water. PDA, buffered PDA, and buffered PDA plus equal amounts of 80% ethanol were used as controls of the in vitro analysis. All experiments were run as duplicates.

#### Preparation of model phenolic acids

Three model phenolic acids (vanillic, p-coumaric, and ferulic acids) that existed in all sorghum grain samples (Leungchaikul 1982) plus cinnamic acid were used for analysis. Cinnamic acid was selected based on its known toxicity to Venturia and ability to inhibit spore germination in some systems (Flood and Kirkham 1960).

The final concentrations of phenolic acids in buffered PDA used for analysis were 1, 4, and 7 mM. The model phenolic acid solutions were prepared in 80% ethanol and incorporated in buffered PDA the same as for extracts (see above).

#### In Vitro analysis

The effects of sorghum non-tannin polyphenols and model phenolic acids on fungal growth were assayed by the agar plug method (Leungchaikul 1982). Pure cultures of each isolate were grown to confluency for 5-6 days on PDA. The agar, containing fungal mycelia, was then removed as small circular plugs with a #6 cork borer (diameter 1.0 cm). The plugs were transferred with a sterile needle and placed, mycelium side down, on the center of the agar plates containing PDA or buffered PDA plus various amounts of sorghum extracts or model phenolic acids. The diameter of mycelial growth (in cm, as the average of three measurements for each plate) was recorded daily over a six day period and plotted vs days of culture.

## RESULTS AND DISCUSSION

### Fungal growth

The fungi most commonly associated with both sorghum samples were Fusarium and Alternaria spp., particularly F. moniliforme, F. semitectum, and A. alternata (Leungchaikul 1982, and Mathur et al 1975). A. flavus, F. moniliforme, and A. alternata were reported to have the greatest potential to produce mycotoxins in sorghum grain (Pettit and Taber 1978). Fusarium isolates were identified as F. semitectum and F. equiseti (see Materials and Methods). Alternaria isolates from sorghum were identified as A. tenuissima complex (see Materials and Methods). The A. tenuissima cpx. has relatively small to medium-size conidia. Some are similar to A. alternata, others to the somewhat larger A. tenuissima (which has additional diagnostic characteristics). F. moniliforme and A. alternata could not be isolated as pure isolates from either of the sorghum samples in the laboratory. Therefore, they were obtained elsewhere (see Materials and Methods). Three isolates of each of F. moniliforme, F. semitectum, F. equiseti, A. alternata, A. tenuissima cpx., and A. flavus were selected for all subsequent in vitro testing. They were numbered as isolates number 1, 2, and 3 for each species for further experimentation.

### Growth curves of fungi

To establish control growth curves and test the agar plug method, all eighteen isolates were grown on PDA and growth was monitored for a six day period. The growth curves of all three isolates of F. moniliforme (Fig. 4), F. semitectum (Fig. 5), F.

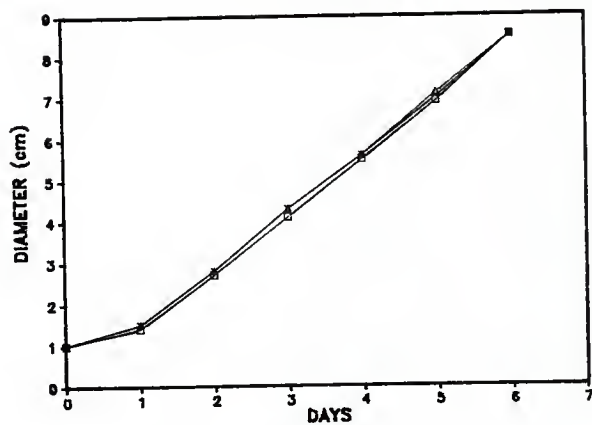
Fig. 4. Growth curves of Fusarium moniliforme isolates.

—□— isolate # 1.  
—X— isolate # 2.  
—△— isolate # 3.

Fig. 5. Growth curves of Fusarium semitectum isolates.

—□— isolate # 1.  
—X— isolate # 2.  
—△— isolate # 3.

*F. moniliforme*



*F. semitectum*

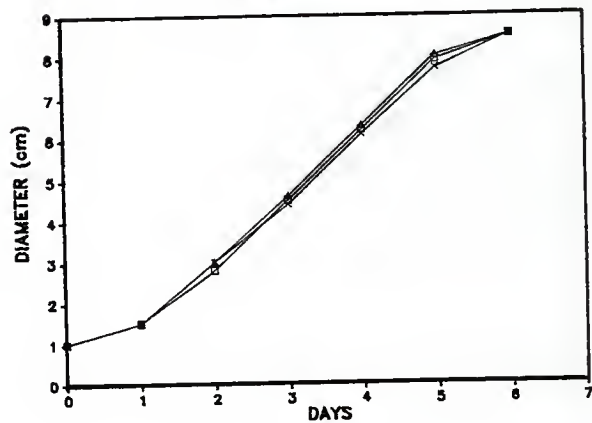


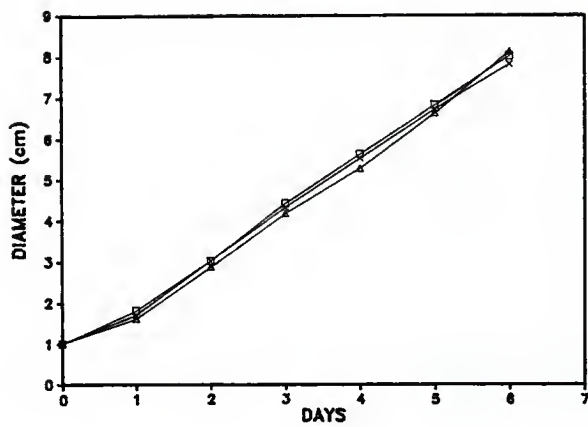
Fig. 6. Growth curves of Fusarium equiseti isolates.

—□— isolate # 1.  
—X— isolate # 2.  
—△— isolate # 3.

Fig. 7. Growth curves of Aspergillus flavus isolates.

—□— isolate # 1.  
—X— isolate # 2.  
—△— isolate # 3.

*F. equiseti*



*A. flavus*

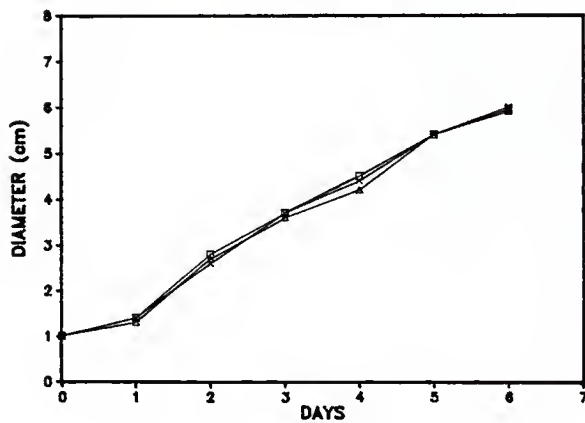


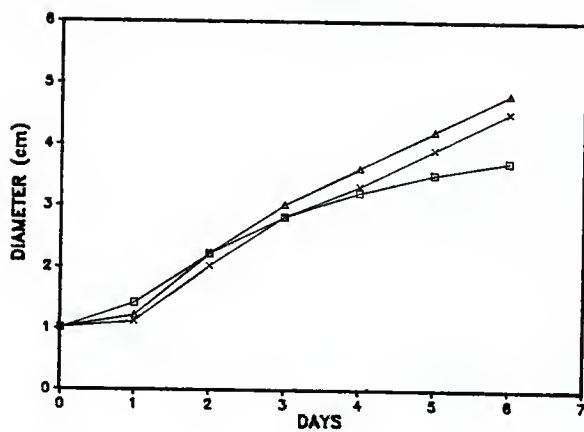
Fig. 8. Growth curves of Alternaria alternata isolates.

—□— isolate # 1.  
—X— isolate # 2.  
—△— isolate # 3.

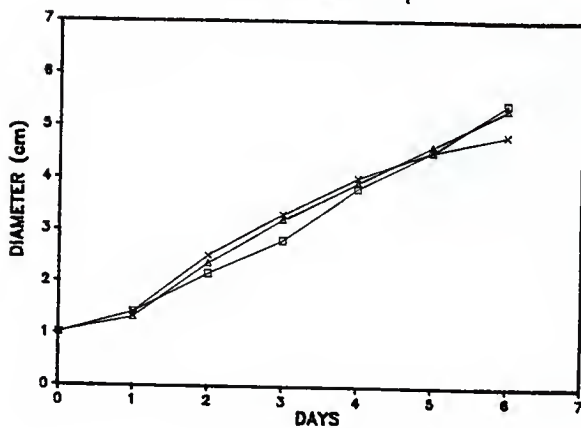
Fig. 9. Growth curves of Alternaria tenuissima complex isolates.

—□— isolate # 1.  
—X— isolate # 2.  
—△— isolate # 3.

*A. alternata*



*A. tenuissima* cpx.



equiseti (Fig. 6), and A. flavus (Fig. 7) were essentially the same. The three isolates of A. alternata (Fig. 8) and A. tenuissima cpx. had different growth rates (Fig. 9) but the patterns of growth were similar. Since growth was essentially similar, only the number 1 isolates of F. moniliforme, F. semitectum, F. equiseti, A. alternata, A. tenuissima cpx., and A. flavus were selected for further in vitro analyses.

#### In vitro Activity of sorghum polyphenols

Extracts of sorghum CS3541 were tested for their activity against fungal isolates. Because the extracts were redissolved in 80% EtOH after evaporation, plates of PDA alone and PDA plus 80% EtOH at equivalent concentrations were used as controls. In the first tests, extracts were added at concentrations of 0.07, 0.29, and 1.02 mg catechin equivalent/ml PDA (mg CE/ml) for the free extract. Appropriate controls contained 0.25, 1.0, and 3.5% (v/v) ethanol, respectively. In the case of the bound extracts, extract concentrations were 0.18, 0.72, and 1.80 mg CE/ml, and controls contained 0.5, 2.0, and 5.0% (v/v) ethanol, respectively.

Preliminary tests showed that both 80% EtOH and pH had minor inhibitory effects on fungal growth. With EtOH, the effect was concentration dependent. Thus, growth inhibition due to phenolic acid extracts must be compared to the appropriate controls. The preliminary test also showed that there was a large decrease in pH of the assay medium at high concentration of the bound extracts. Therefore, the highest concentration control plate (5% v/v) was treated with both 80% EtOH and tartaric acid (to control pH) in the same plate. Mycelial growth was measured three times for each

plate with duplications of each concentration. The results are shown in Table A and B (Appendix) for the free and bound extracts respectively. The data shown in the table are averages of six day 6 readings  $\pm$  a 95% confidence interval for each. The numbers are presented in a form  $\bar{X} \pm t_{0.025} S/\sqrt{n}$  with a d.f. = 5 or  $\bar{X} \pm S(1.05)$ .

To assess the effect of extract presence on fungal growth, growth on each plate was compared graphically to growth on control plates (PDA alone, PDA plus 80% EtOH at equivalent concentrations or PDA plus 80% EtOH and tartaric acid at equivalent concentrations and pH). Thus, there are four graphs for each fungal isolate. The graphs represent the growth curves of fungi on all concentration of extracts and their controls (shown in graph A) and each concentration (low, medium, and high concentration) of extract and appropriate controls (shown in graph B, C, and D respectively).

No growth inhibition of the three Fusarium spp. (Figs. 10-12) and A. flavus (Fig. 15) could be detected at any free extract concentration. To the contrary, a marked of stimulation of growth was observed at concentration 0.07 mg CE/ml. For Alternaria spp. (Figs. 13 and 14), inhibition occurred by day 2 at 0.07 mg CE/ml (Figs. 13B and 14B). But at higher concentration, inhibition was reduced (Figs. 13C and 14C&D) and growth actually was stimulated at high concentration (1.02 mg CE/ml) (Fig. 13D) for A. alternata. However, significant inhibition was detected for all fungal isolates at any bound extract concentration, except at 0.18 mg CE/ml for A. flavus (Figs 16-21). Complete growth inhibition for all fungi occurred from 0.72 mg CE/ml, but occurred only at 1.80 mg CE/ml for A. Flavus. Growth inhibition started at either day 1 or

day 2 and continued to day 6 at all tested concentrations.

The bound extract decreased the pH of medium, whereas the free extract results in a slight increase in pH. Therefore, the observed results could have been due to either the phenolic acids in the extract or pH effects. Phosphate buffer (0.2 M, pH 6.4) was then used in place of distilled water in PDA to minimize differences in pH, but the problem was not overcome for the highest concentrations of bound extract. Higher concentrations of phosphate buffer did not solve the problem because the PDA media formed a very hard gel and fungi did not grow well due to its high ionic strength. Therefore, 0.2 M phosphate buffer solution (pH 6.4) was used in subsequent experiments. Tartaric acid was also added to control pH of the high concentration of the bound extract as used previously.

Free and bound extracts of sorghum SC0719 were tested for their activity against fungal isolates in buffered PDA at 1.65, 6.60, and 16.5 mg CE/ml (free extract) and at 2.33, 9.30, and 23.3 mg CE/ml (bound extract). This is equivalent to 0.5, 2.0, and 5.0% v/v. The results are shown in Table C and D (Appendix). The effects of SC0719 free extract against the six fungi are graphically presented in Figs. 22-27. Slight inhibition was observed at 1.65 mg CE/ml against *E. moniliforme* (Fig. 22B), and *A. tenuissima* cpx. (Fig. 26B), and at 6.60 mg CE/ml against *E. semitectum* (Fig. 23C). The differences, while small, can be considered significant due to lack of overlap of the confidence intervals. At high concentration, growth stimulation occurred at day 1 through day 6 for all fungi (Figs. 22-27). On the other hand, the bound extract from SC0719 inhibited growth of all three *Fusarium* spp. from day 2 to day 6 at

any concentration (Figs. 28-30). However, only 23.3 mg CE/ml inhibited growth of both Alternaria spp. and A. flavus (Figs. 31-33). Complete inhibition occurred at 23.3 mg CE/ml for all fungal isolates except A. flavus.

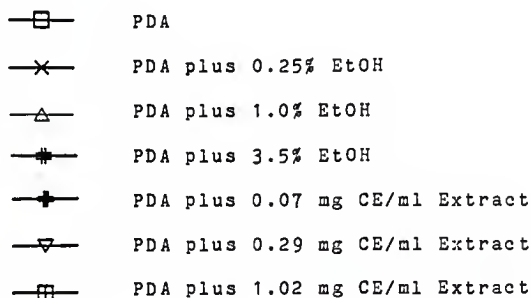
Extracts of sorghum CS3541 were also retested again in buffered PDA at 0.02, 0.1, and 0.2 mg CE/ml (free extract) (0.3, 1.5, and 3.0% v/v). For the bound extract, experiments were carried out at 0.16, 0.37, and 0.79 mg CE/ml (0.3, 0.7, and 1.5% v/v). Because the 80% EtOH used as solvent had slight inhibitory effects on fungal growth (Figs. 16D-21D), the experiment was done by using the absolute minimum amount of 80% EtOH.

The results are shown in Table E and F (Appendix). No inhibition of any fungus, except F. equiseti, was detected at any free extract concentration (Fig. 36B). Little inhibition was observed at solely 0.02 mg CE/ml, by day 4. In contrast, growth stimulation of all fungi occurred at day 2 at 0.2 mg CE/ml (Fig. 34-39). In the case of the bound extract, growth inhibition was detected for all six fungi (Figs. 40-45). At any concentration, the CS3541 bound extract inhibited F. moniliforme as early as day 1 (Fig. 40B-D). For F. semitectum and F. equiseti, growth inhibition started later, by day 3 at 0.16 mg CE/ml (Figs. 41B and 42B). At 0.37 mg CE/ml (Fig. 41C), F. semitectum was inhibited by day 2 but no inhibition was observed for F. equiseti (Fig. 42C). No inhibition was detected at 0.16 mg CE/ml for Alternaria spp. (Figs. 43B and 44B). However, the bound extract inhibited Alternaria spp. by day 1 at 0.37 mg CE/ml (Figs. 43C and 44C). No inhibition for A. flavus was observed at concentrations 0.16 and 0.37 mg CE/ml (Figs. 45B & 45C). At 0.79 mg CE/ml, inhibition of all fungi started at

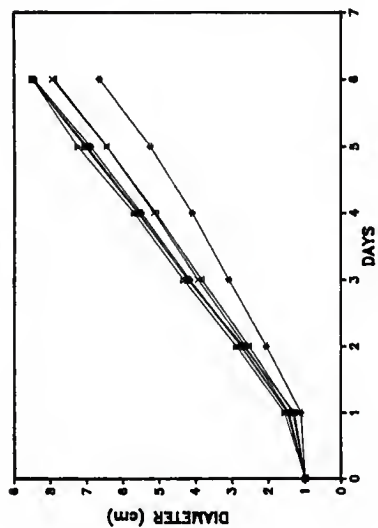
day 1, except for *A. flayus* which started at day 2 (Figs. 40D-45D). Complete inhibition occurred at 0.79 mg CE/ml for *F. semitectum*, *A. alternata*, and *A. tenuissima* opx.

The results of the *in vitro* tests determined that bound extracts of both sorghum CS3541 and SC0719 inhibited growth of all six fungi. Unexpectedly, the bound extract from CS3541 appeared to be more effective than that from SC0719. It was observed that CS3541 and SC0719 bound extracts had different inhibitory effects on the growth of individual fungal species. Furthermore, the inhibitory effects were different for different genus of fungus with regard to onset and extent of growth inhibition. Thus, the extent of inhibition and minimum inhibitory concentration depends upon the fungus. Surprisingly, free extracts of both sorghums showed a growth stimulatory effect for all six fungi. The stimulation of the growth of other fungi by other phenolic acids in other plant has been observed previously (Farkas and Kiraly 1962, Friend 1977, and Van Sumere 1960). As with the inhibitory effects of bound extracts, the stimulatory activity of the free extracts appears to depend upon individual fungus and sorghum variety.

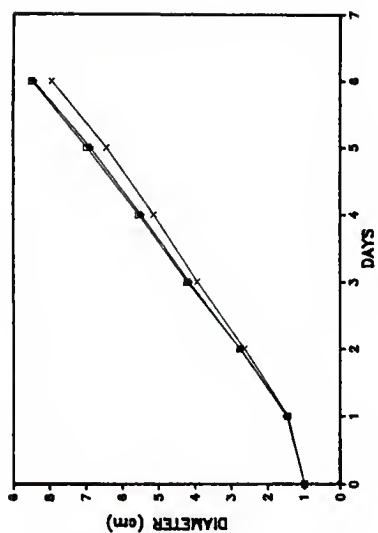
Fig. 10. Diameter of mycelial growth vs. days of culture for *F. moniliforme* against CS3541 free extract and controls.



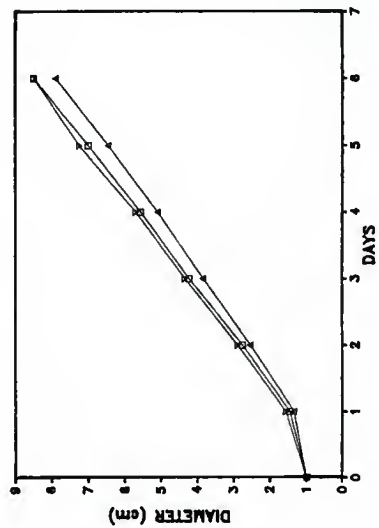
A



B



C



D

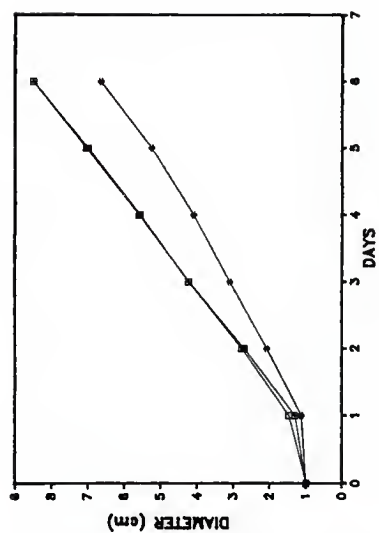


Fig. 11. Diameter of mycelial growth vs. days of culture for F. semitectum against CS3541 free extract and controls.

—□—	PDA
—X—	PDA plus 0.25% EtOH
—△—	PDA plus 1.0% EtOH
—≡—	PDA plus 3.5% EtOH
—◇—	PDA plus 0.07 mg CE/ml Extract
—+—	PDA plus 0.29 mg CE/ml Extract
—▽—	PDA plus 1.02 mg CE/ml Extract

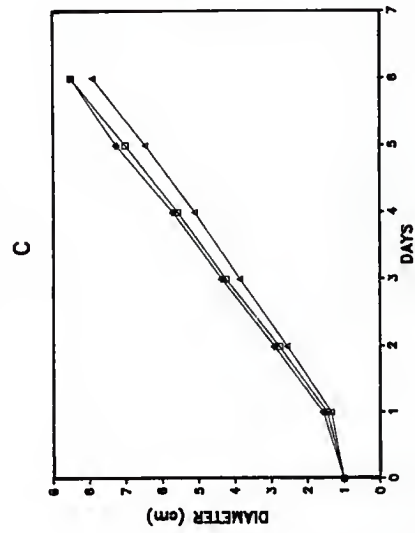
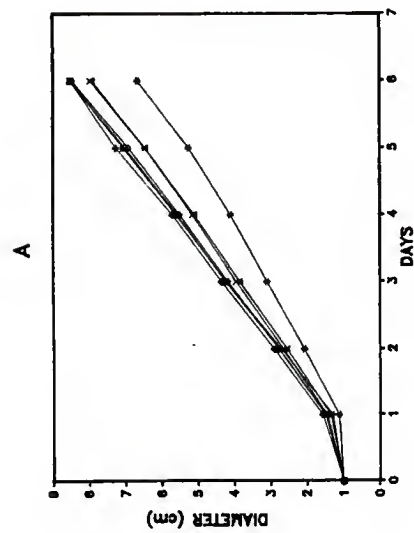
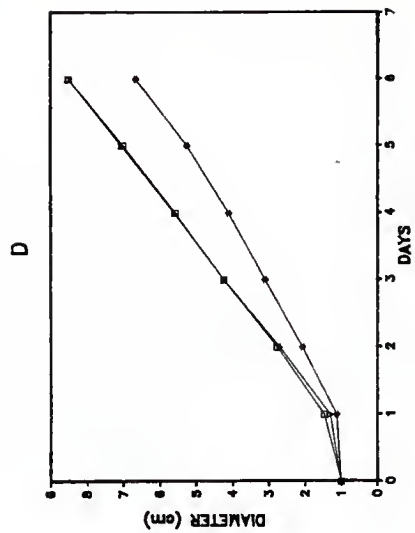
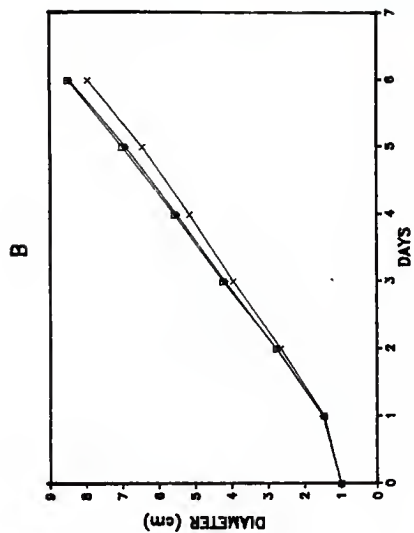
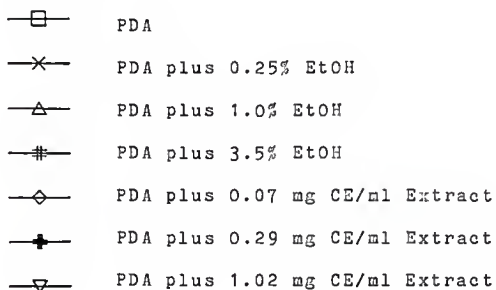
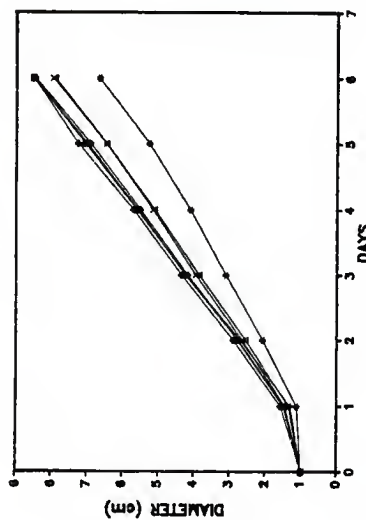


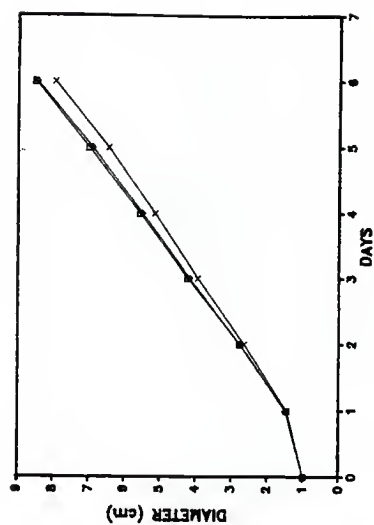
Fig. 12. Diameter of mycelial growth vs. days of culture for F. equiseti against CS3541 free extract and controls.



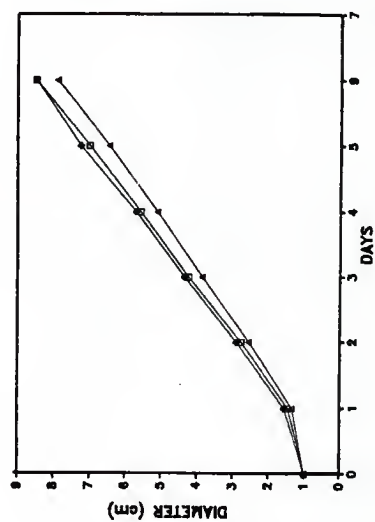
A



B



C



D

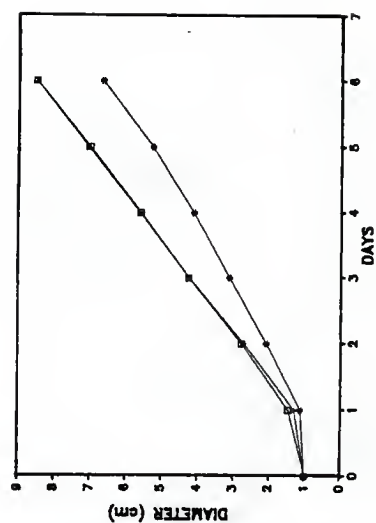
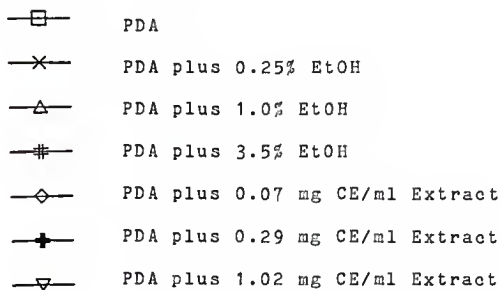
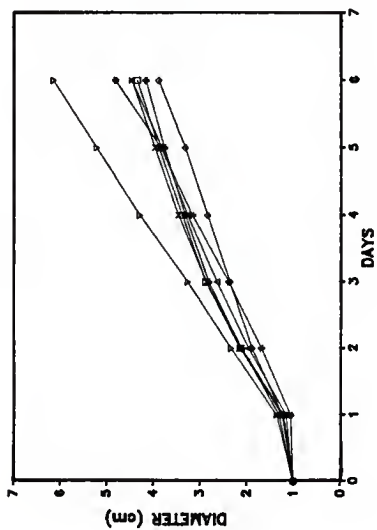


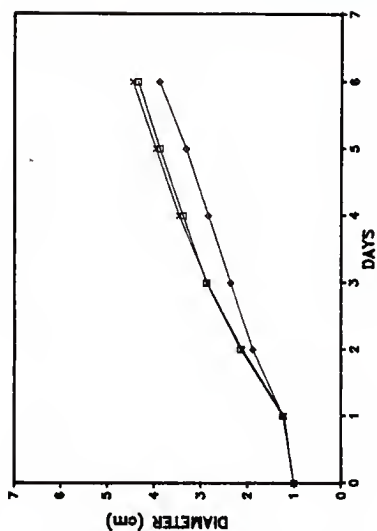
Fig. 13. Diameter of mycelial growth vs. days of culture for A. alternata against CS3541 free extract and controls.



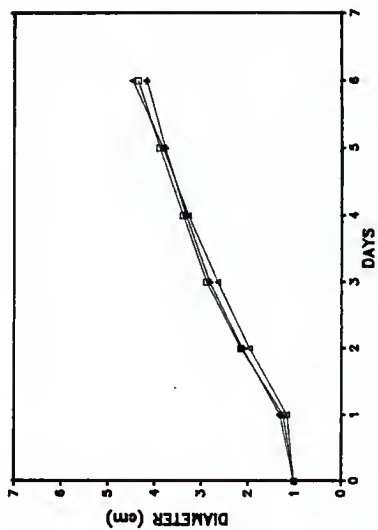
A



B



C



D

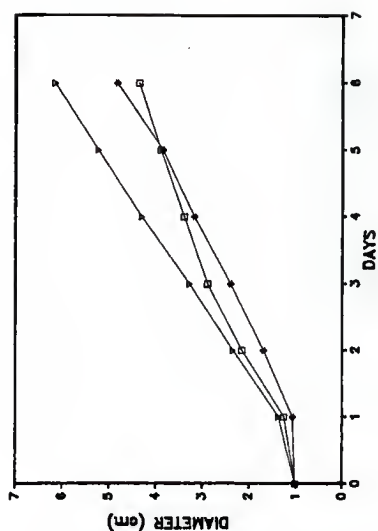







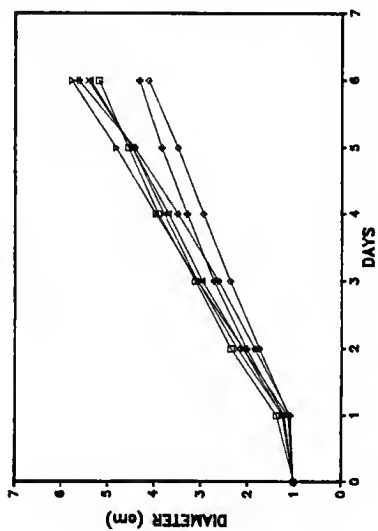


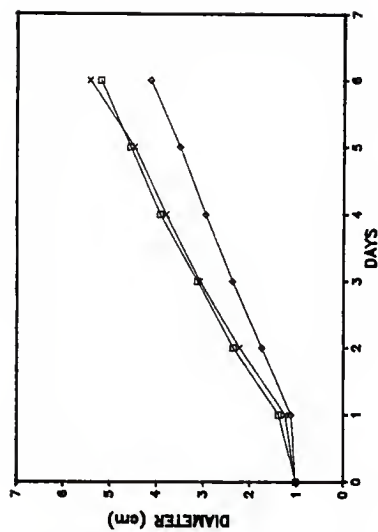
Fig. 14. Diameter of mycelial growth vs. days of culture for A. tenuissina cpx. against CS3541 free extract and controls.

	PDA
	PDA plus 0.25% EtOH
	PDA plus 1.0% EtOH
	PDA plus 3.5% EtOH
	PDA plus 0.07 mg CE/ml Extract
	PDA plus 0.29 mg CE/ml Extract
	PDA plus 1.02 mg CE/ml Extract

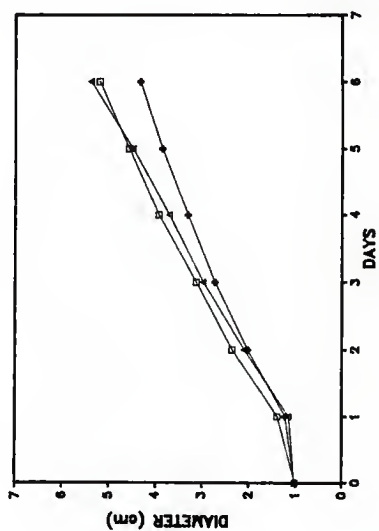
A



B



C



D

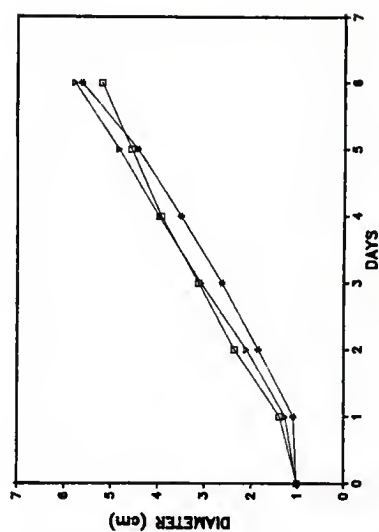
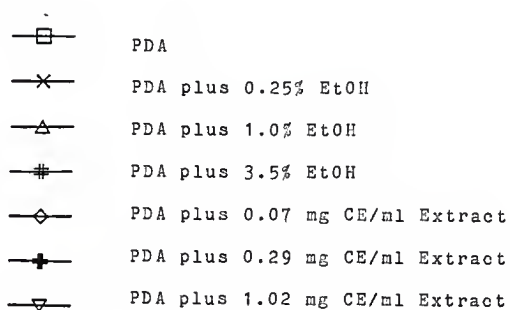


Fig. 15. Diameter of mycelial growth vs. days of culture for F. flavus against CS3541 free extract and controls.



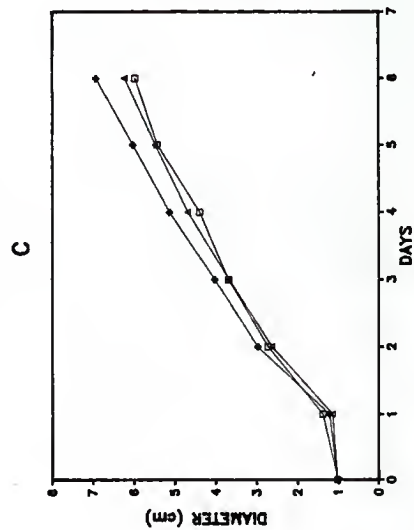
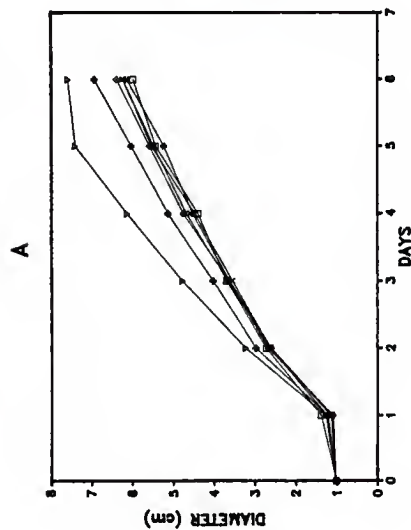
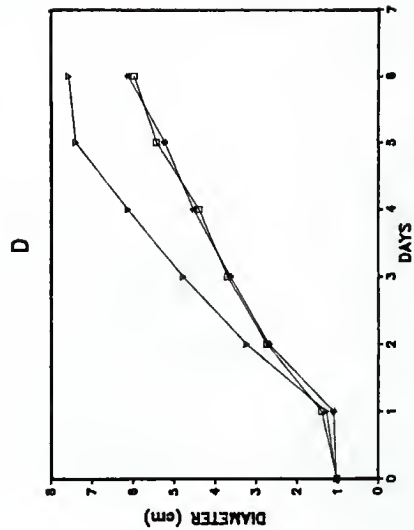
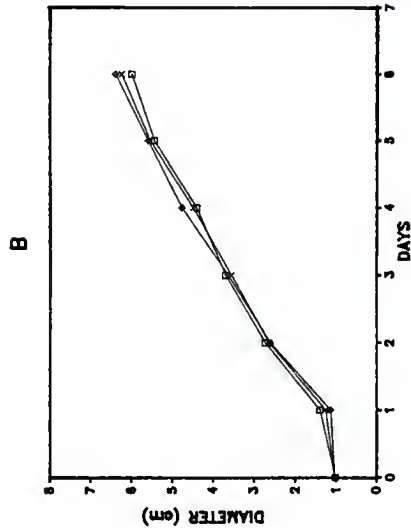
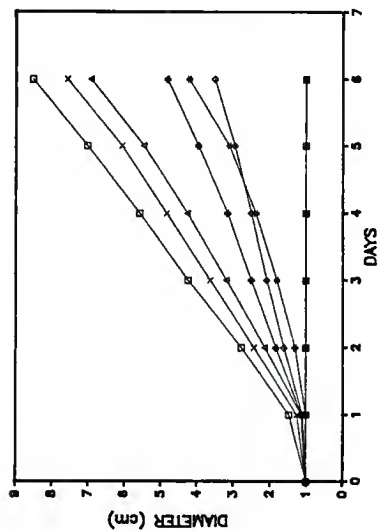


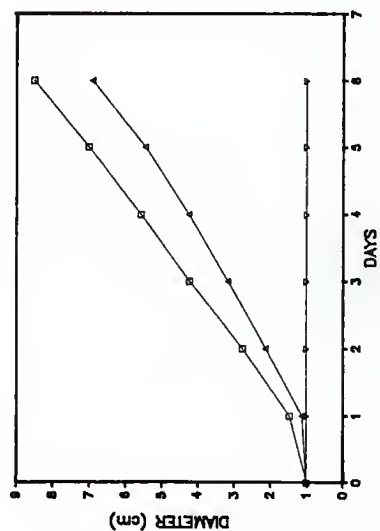
Fig. 16. Diameter of mycelial growth vs. days of culture for E. moniliiforme against CS3541 bound extract and controls.

—□—	PDA
—X—	PDA plus 0.5% EtOH
—△—	PDA plus 2.0% EtOH
—#—	PDA plus 5.0% EtOH
—◇—	PDA plus 5.0% EtOH plus tartaric acid (same pH as —▣— )
—+—	PDA plus 0.18 mg CE/ml Extract
—▽—	PDA plus 0.72 mg CE/ml Extract
—▣—	PDA plus 1.80 mg CE/ml Extract

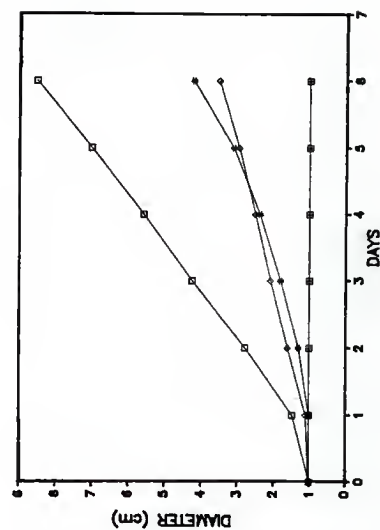
A



C



D



B

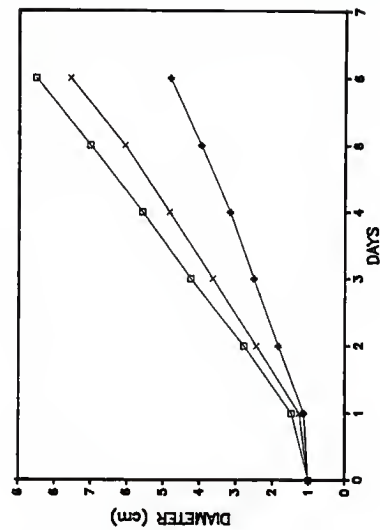


Fig. 17. Diameter of mycelial growth vs. days of culture for *F. semitectum* against CS3541 bound extract and controls.

—□—	PDA
—X—	PDA plus 0.5% EtOH
—△—	PDA plus 2.0% EtOH
—#—	PDA plus 5.0% EtOH
—◇—	PDA plus 5.0% EtOH plus tartaric acid (same pH as —■— )
—+—	PDA plus 0.18 mg CE/ml Extract
—▽—	PDA plus 0.72 mg CE/ml Extract
—■—	PDA plus 1.80 mg CE/ml Extract

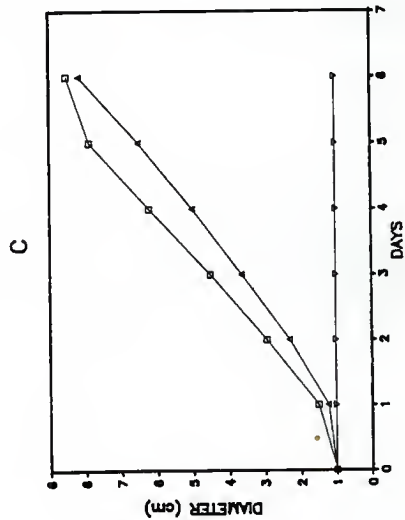
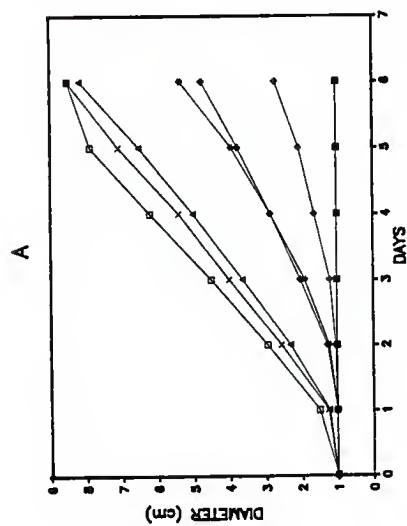
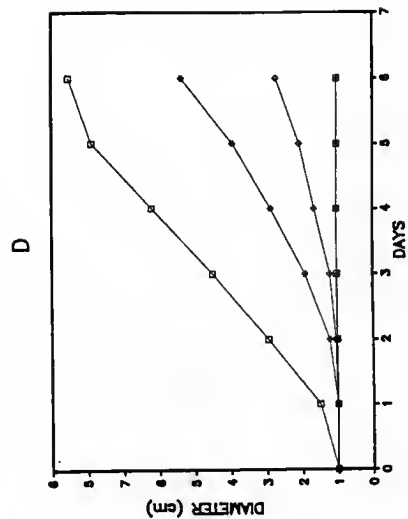
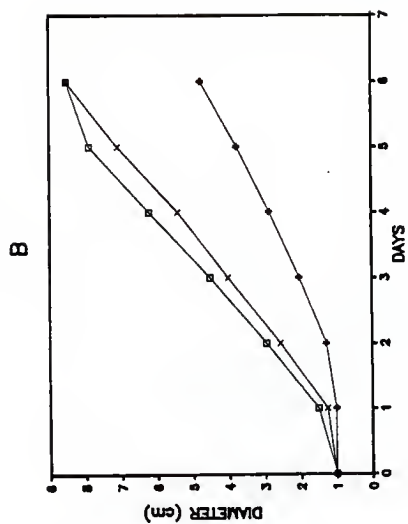
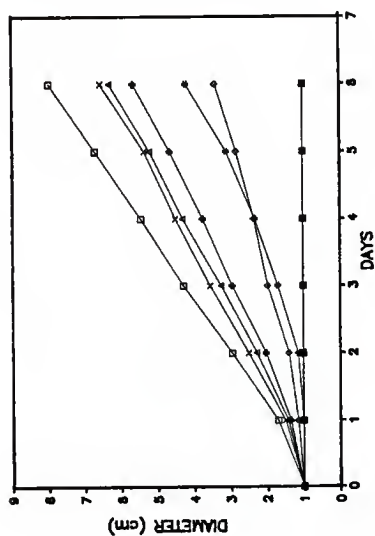


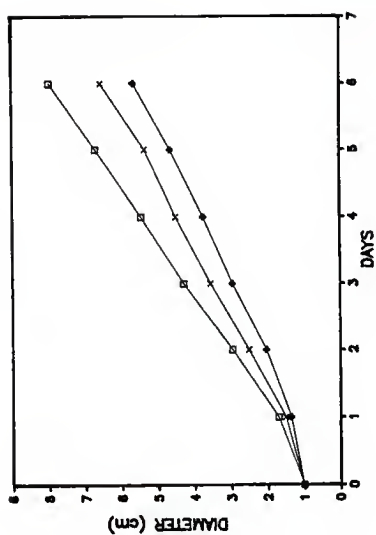
Fig. 18. Diameter of mycelial growth vs. days of culture for F. equiseti against CS3541 bound extract and controls.

—□—	PDA
—X—	PDA plus 0.5% EtOH
—△—	PDA plus 2.0% EtOH
—#—	PDA plus 5.0% EtOH
—◇—	PDA plus 5.0% EtOH plus tartaric acid (same pH as —■— )
—+—	PDA plus 0.18 mg CE/ml Extract
—▽—	PDA plus 0.72 mg CE/ml Extract
—■—	PDA plus 1.80 mg CE/ml Extract

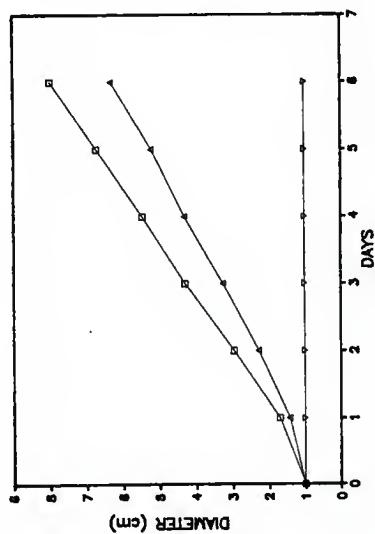
A



B



C



D

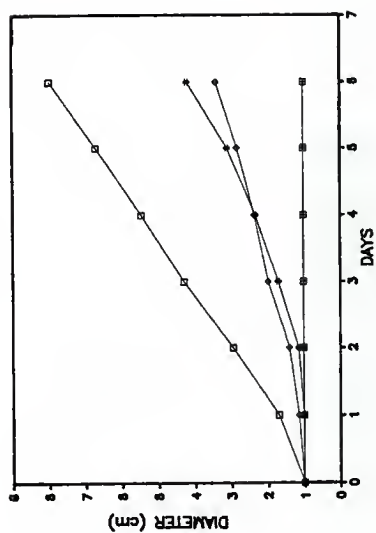
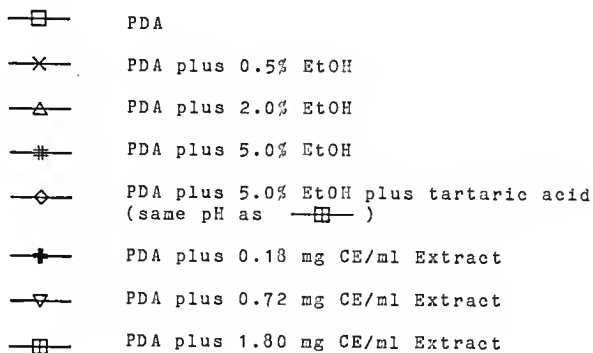
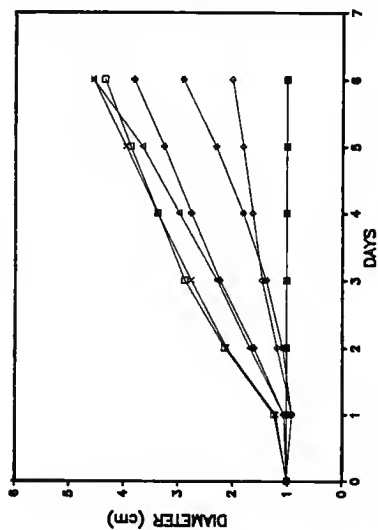


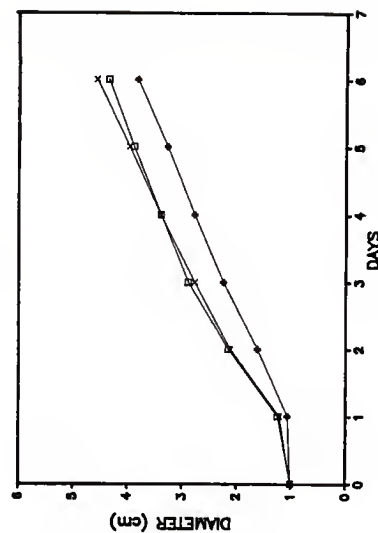
Fig. 19. Diameter of mycelial growth vs. days of culture for A. alternata against CS3541 bound extract and controls.



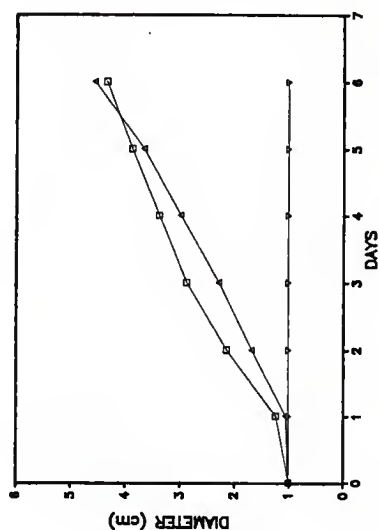
A



B



C



D

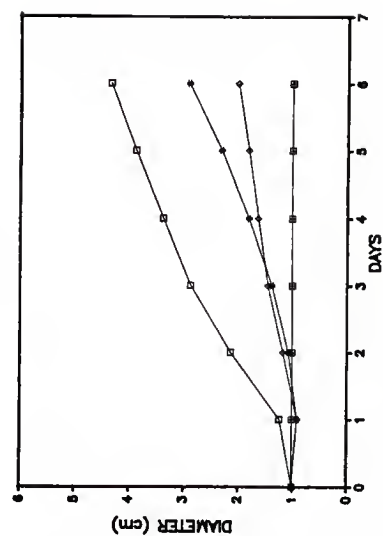









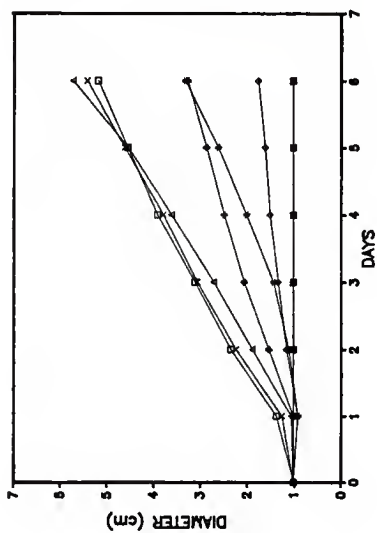


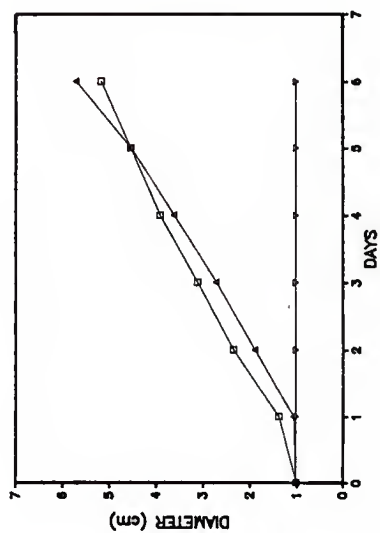
Fig. 20. Diameter of mycelial growth vs. days of culture for A. tenuissima opx. against CS3541 bound extract and controls.

	PDA
	PDA plus 0.5% EtOH
	PDA plus 2.0% EtOH
	PDA plus 5.0% EtOH
	PDA plus 5.0% EtOH plus tartaric acid (same pH as  )
	PDA plus 0.18 mg CE/ml Extract
	PDA plus 0.72 mg CE/ml Extract
	PDA plus 1.80 mg CE/ml Extract

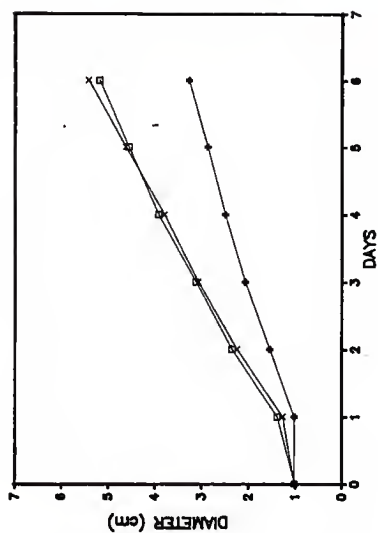
A



C



B



D

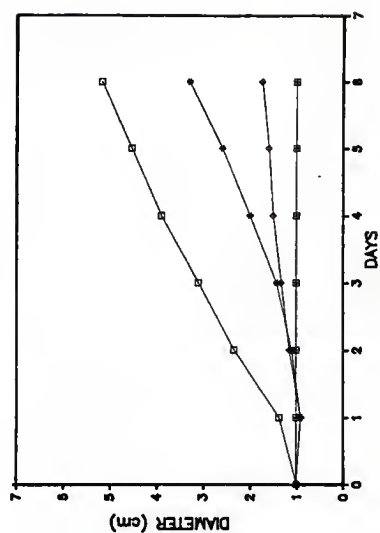
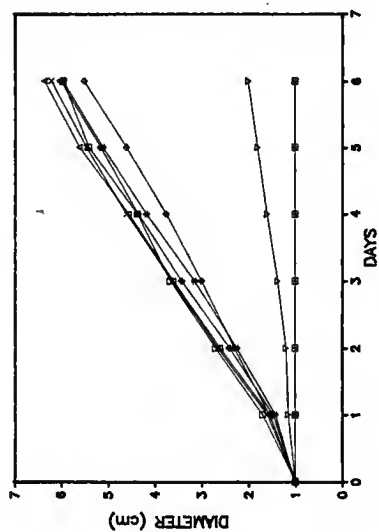


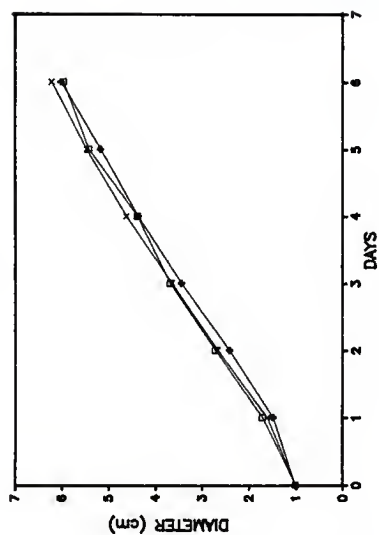
Fig. 21. Diameter of mycelial growth vs. days of culture for  
A. flavus against CS3541 bound extract and controls.

—□—	PDA
—X—	PDA plus 0.5% EtOH
—△—	PDA plus 2.0% EtOH
—#—	PDA plus 5.0% EtOH
—◇—	PDA plus 5.0% EtOH plus tartaric acid (same pH as —▣— )
—+—	PDA plus 0.18 mg CE/ml Extract
—▽—	PDA plus 0.72 mg CE/ml Extract
—▣—	PDA plus 1.80 mg CE/ml Extract

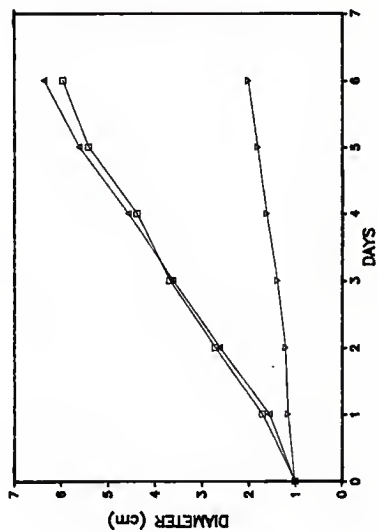
A



B



C



D

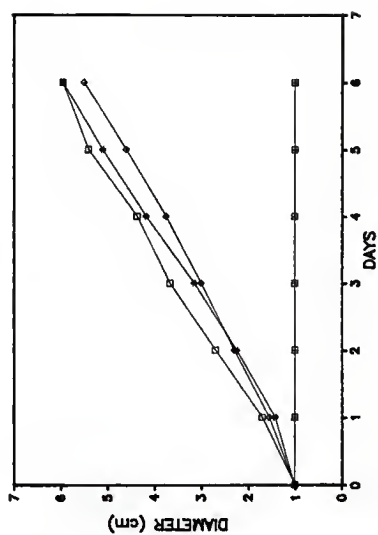


Fig. 22. Diameter of mycelial growth vs. days of culture for F. moniliforme against SC0719 free extract and controls.

<del>—□—</del>	PDA
<del>—×—</del>	BPDA (Buffered PDA)
<del>—△—</del>	BPDA plus 0.5% EtOH
<del>—#—</del>	BPDA plus 2.0% EtOH
<del>—◇—</del>	BPDA plus 5.0% EtOH
<del>—+—</del>	BPDA plus 1.65 mg CE/ml Extract
<del>—▽—</del>	BPDA plus 6.60 mg CE/ml Extract
<del>—⊠—</del>	BPDA plus 16.5 mg CE/ml Extract

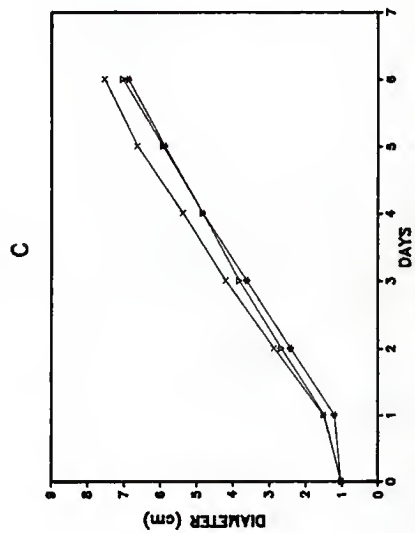
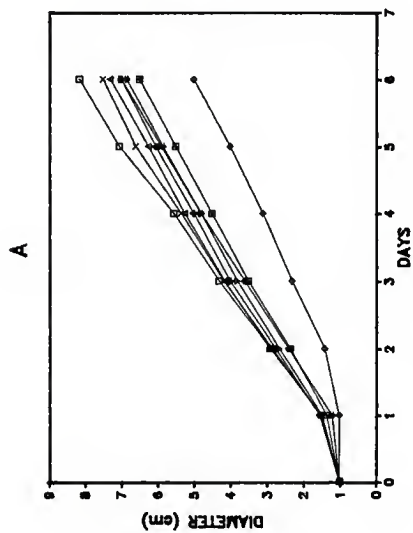
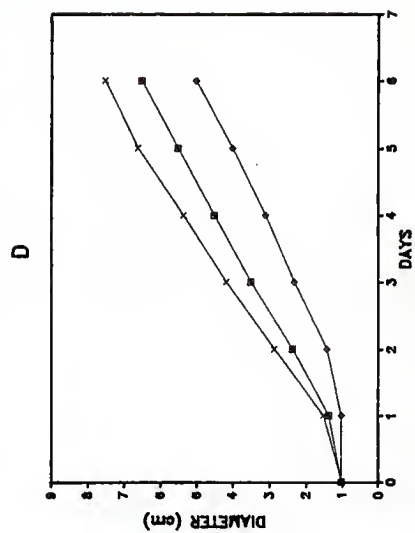
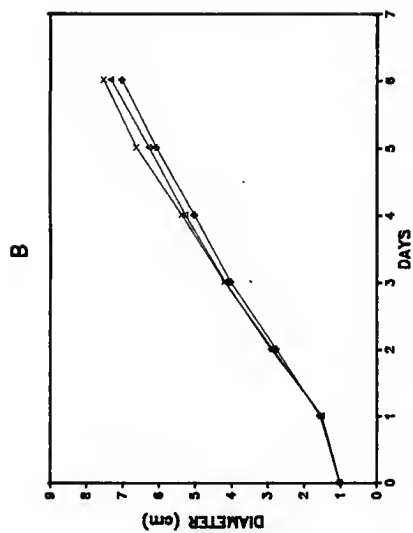
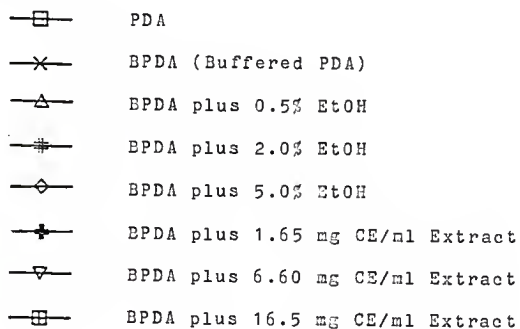


Fig. 23. Diameter of mycelial growth vs. days of culture for F. semitectum against SC0719 free extract and controls.



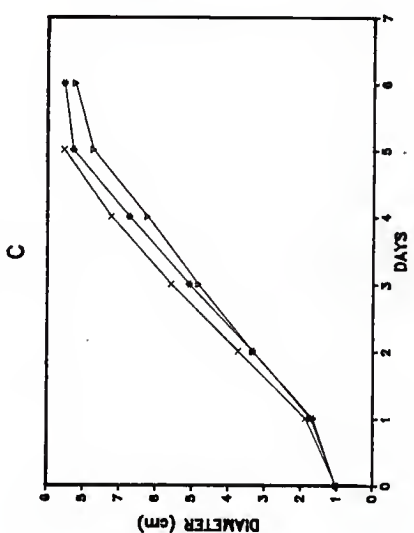
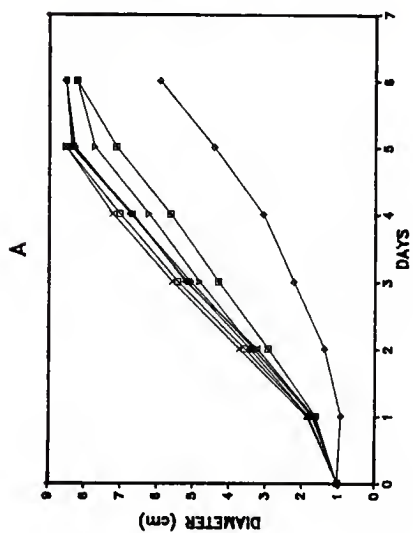
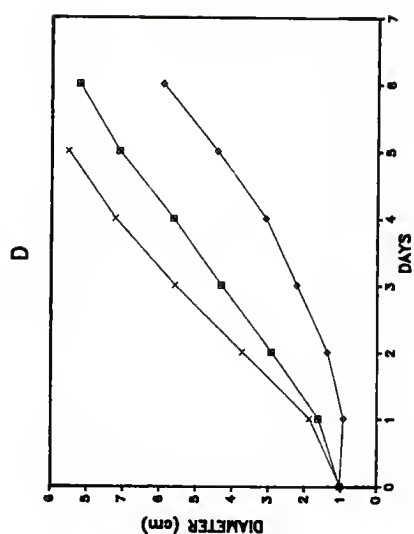
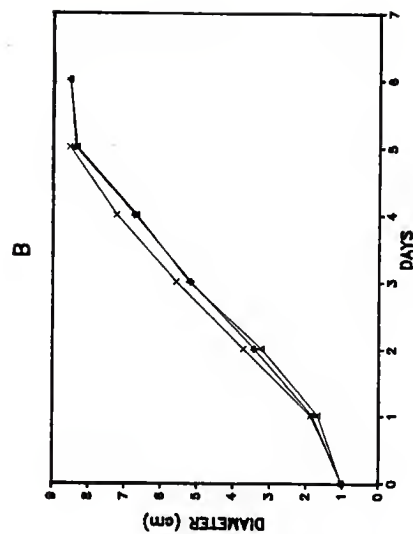










Fig. 24. Diameter of mycelial growth vs. days of culture for E. equiseti against SC0719 free extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.5% EtOH
	BPDA plus 2.0% EtOH
	BPDA plus 5.0% EtOH
	BPDA plus 1.65 mg CE/ml Extract
	BPDA plus 6.60 mg CE/ml Extract
	BPDA plus 16.5 mg CE/ml Extract

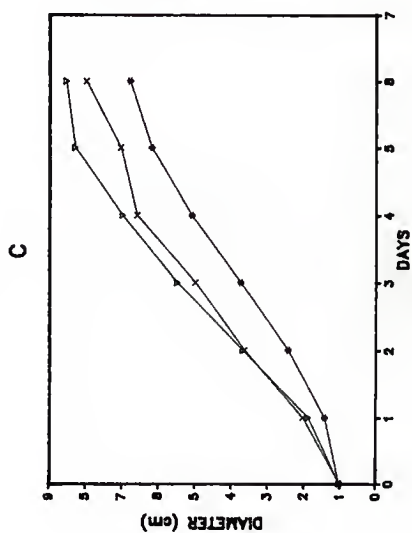
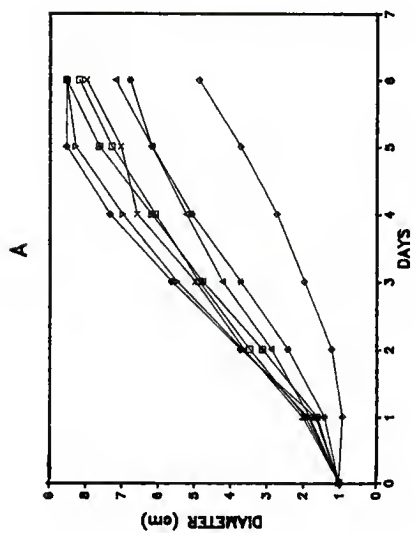
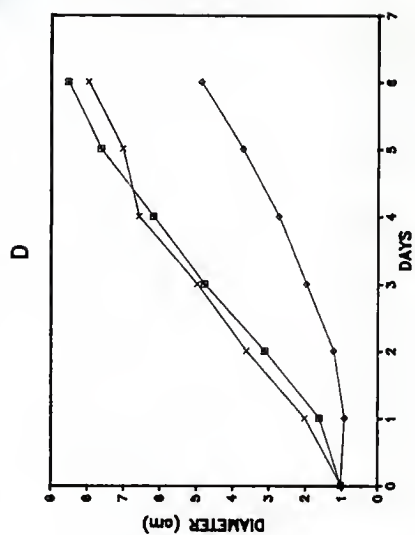
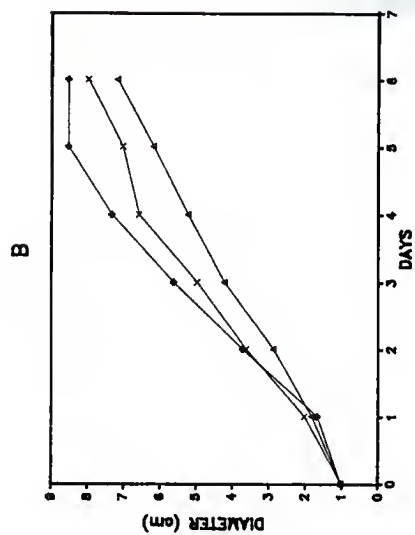
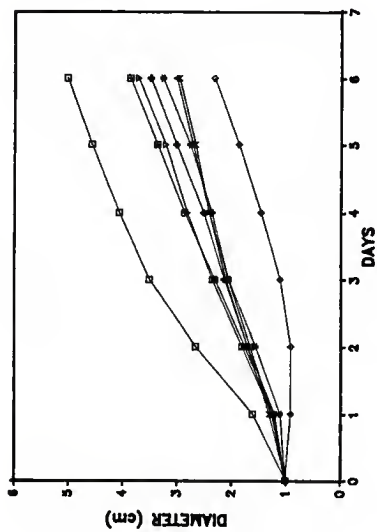


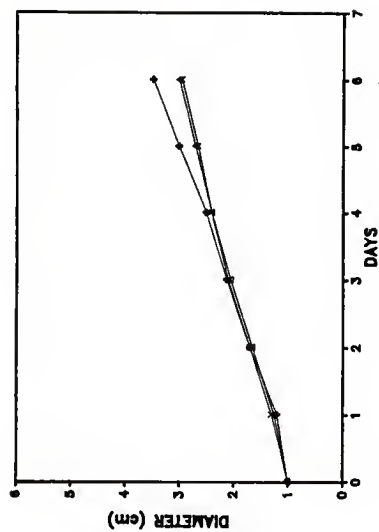
Fig. 25. Diameter of mycelial growth vs. days of culture for A. alternata against SC0719 free extract and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 5.0% EtOH
—+—	BPDA plus 1.65 mg CE/ml Extract
—▽—	BPDA plus 6.60 mg CE/ml Extract
—■—	BPDA plus 16.5 mg CE/ml Extract

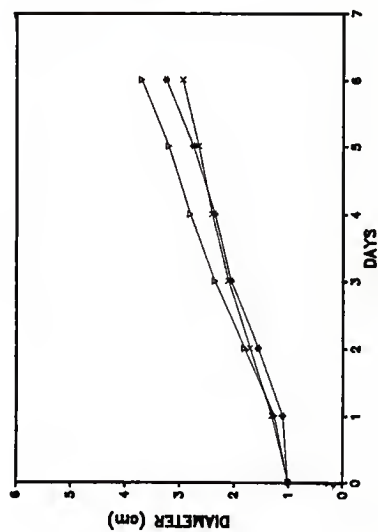
A



B



C



D

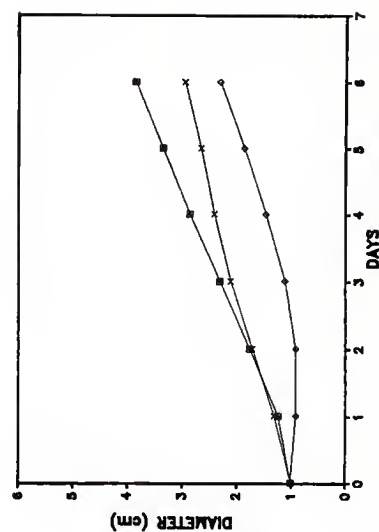










Fig. 26. Diameter of mycelial growth vs. days of culture for A. tenuissima cpx. against SC0719 free extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.5% EtOH
	BPDA plus 2.0% EtOH
	BPDA plus 5.0% EtOH
	BPDA plus 1.65 mg CE/ml Extract
	BPDA plus 6.60 mg CE/ml Extract
	BPDA plus 16.5 mg CE/ml Extract

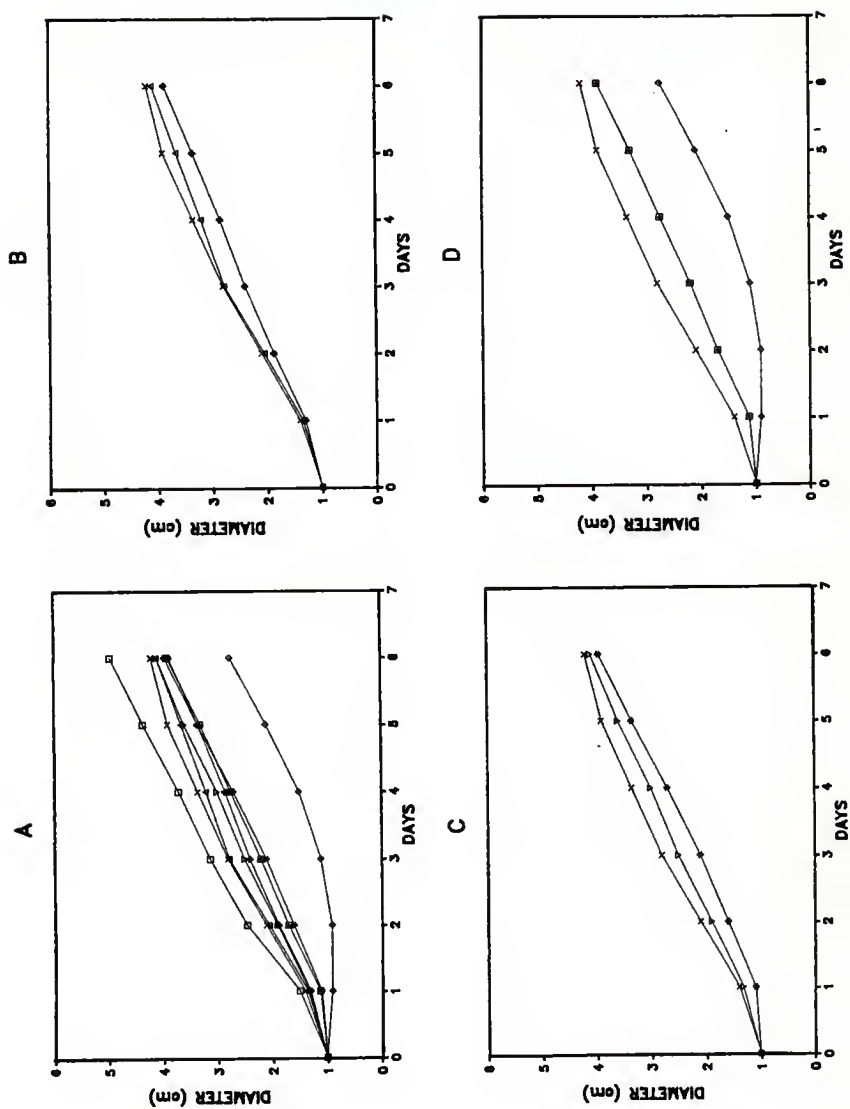


Fig. 27. Diameter of mycelial growth vs. days of culture for *A. flavus* against SC0719 free extract and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 5.0% EtOH
—+—	BPDA plus 1.65 mg CE/ml Extract
—▽—	BPDA plus 6.60 mg CE/ml Extract
—⊠—	BPDA plus 16.5 mg CE/ml Extract

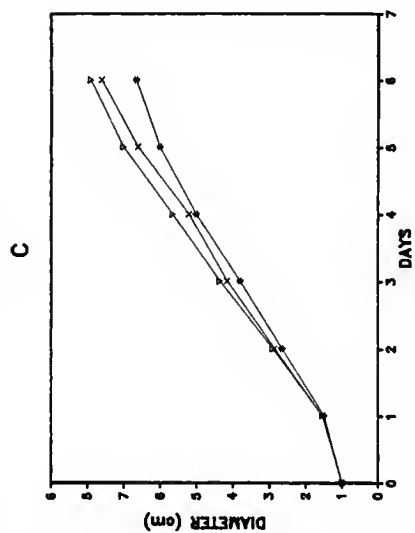
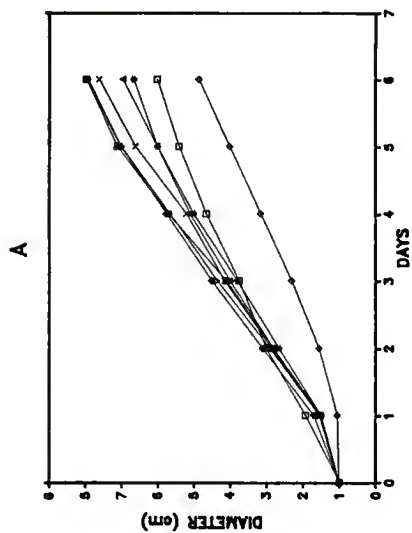
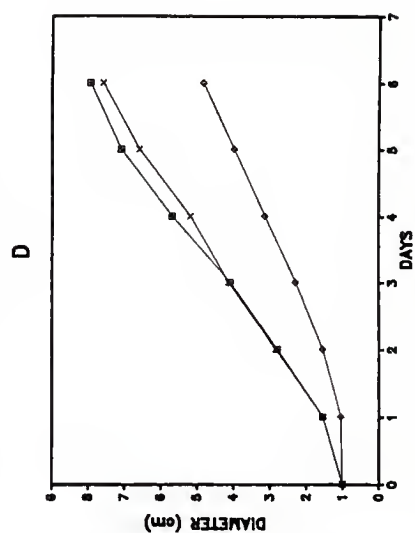
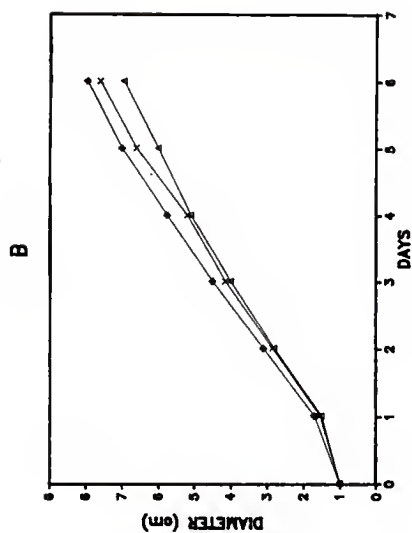












Fig. 28. Diameter of mycelial growth vs. days of culture for F. moniliforme against SC0719 bound extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.5% EtOH
	BPDA plus 2.0% EtOH
	BPDA plus 5.0% EtOH
	BPDA plus 5.0% EtOH plus tartaric acid (same pH as  )
	BPDA plus 2.33 mg CE/ml Extract
	BPDA plus 9.30 mg CE/ml Extract
	BPDA plus 23.3 mg CE/ml Extract

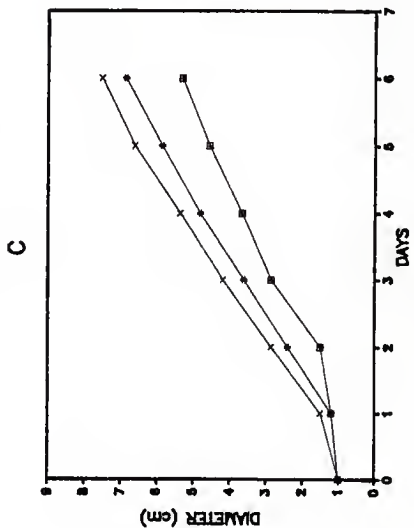
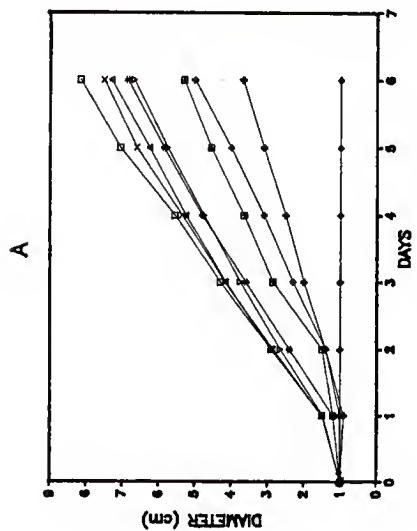
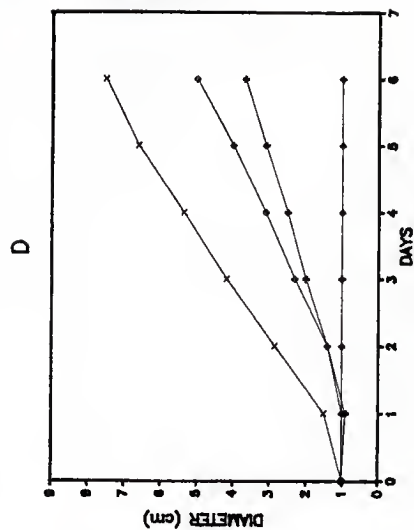
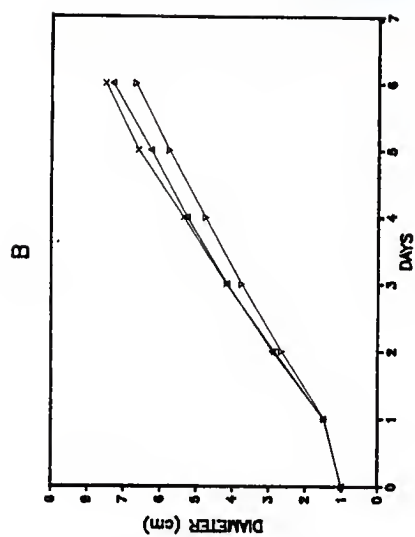
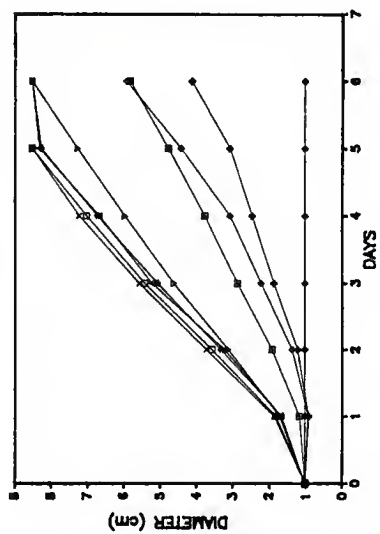


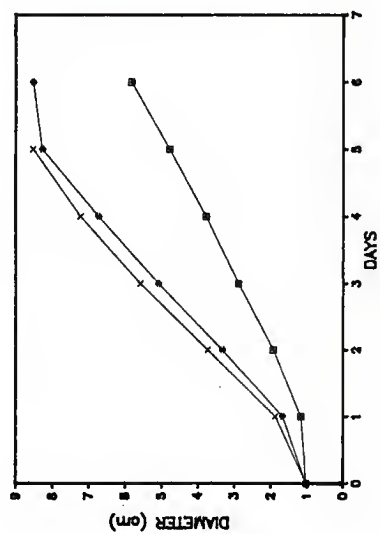
Fig. 29. Diameter of mycelial growth vs. days of culture for *F. semitectum* against SC0719 bound extract and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 5.0% EtOH
—+—	BPDA plus 5.0% EtOH plus tartaric acid (same pH as —◆— )
—▽—	BPDA plus 2.33 mg CE/ml Extract
—⊠—	BPDA plus 9.30 mg CE/ml Extract
—◆—	BPDA plus 23.3 mg CE/ml Extract

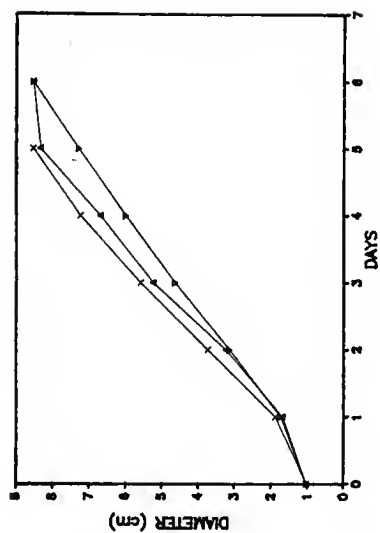
A



C



B



D

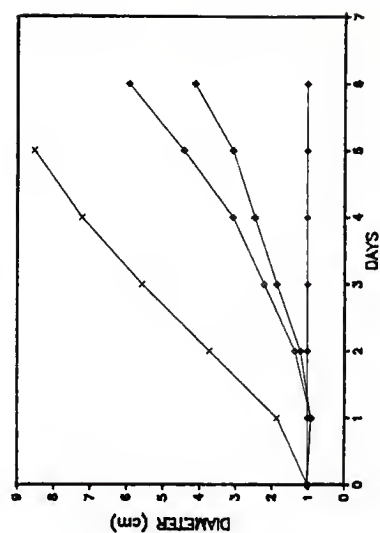












Fig. 30. Diameter of mycelial growth vs. days of culture for E. equiseti against SC0719 bound extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.5% EtOH
	BPDA plus 2.0% EtOH
	BPDA plus 5.0% EtOH
	BPDA plus 5.0% EtOH plus tartaric acid (same pH as  )
	BPDA plus 2.33 mg CE/ml Extract
	BPDA plus 9.30 mg CE/ml Extract
	BPDA plus 23.3 mg CE/ml Extract

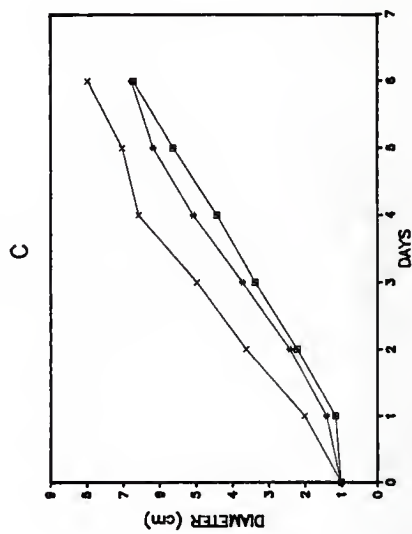
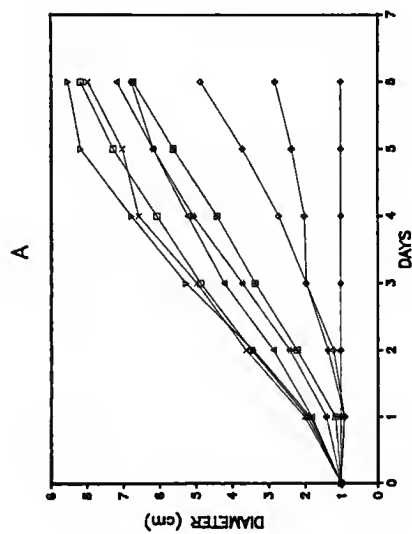
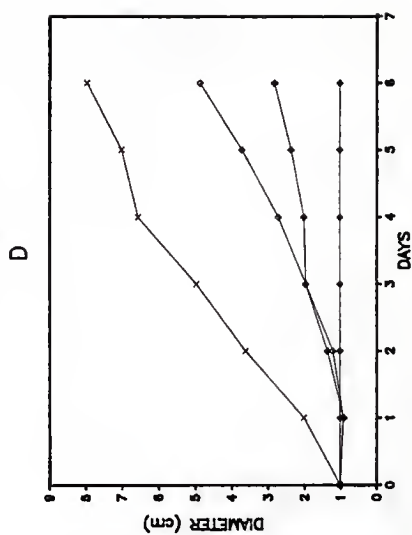
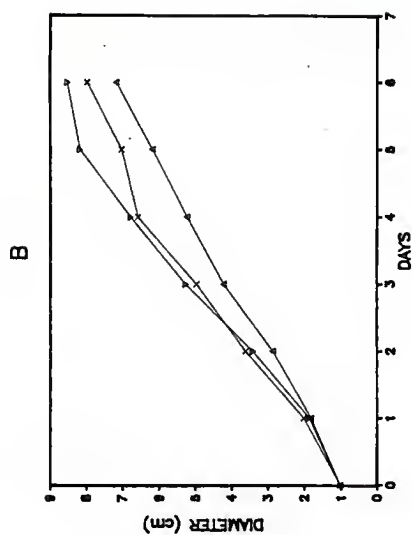
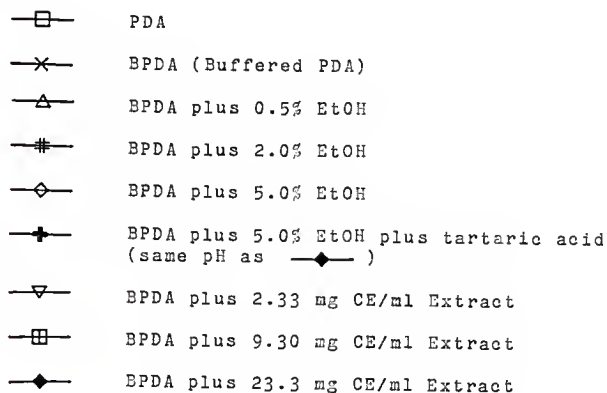


Fig. 31. Diameter of mycelial growth vs. days of culture for A. alternata against SC0719 bound extract and controls.



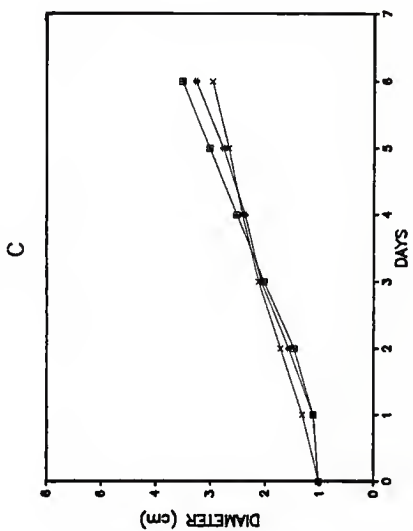
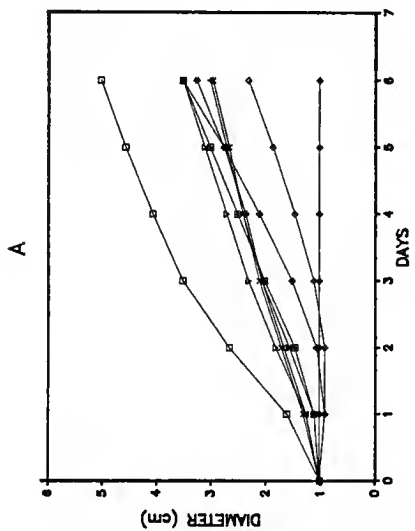
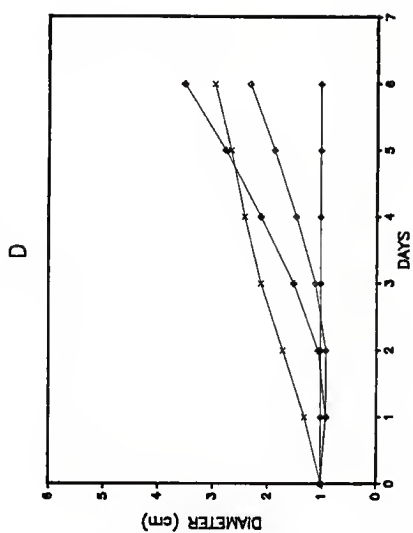
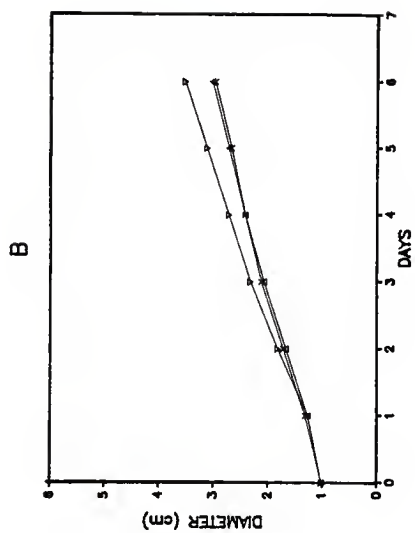












Fig. 32. Diameter of mycelial growth vs. days of culture for A. tenuissina cpx. against SC0719 bound extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.5% EtOH
	BPDA plus 2.0% EtOH
	BPDA plus 5.0% EtOH
	BPDA plus 5.0% EtOH plus tartaric acid (same pH as  )
	BPDA plus 2.33 mg CE/ml Extract
	BPDA plus 9.30 mg CE/ml Extract
	BPDA plus 23.3 mg CE/ml Extract

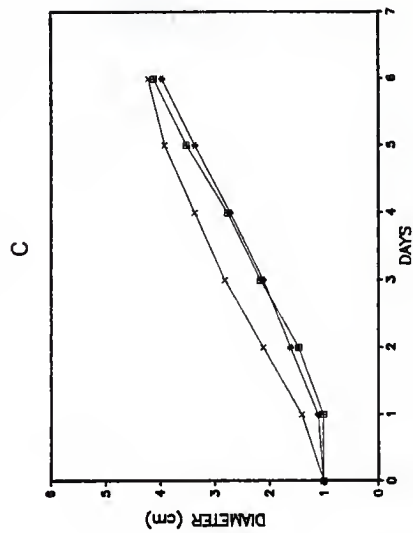
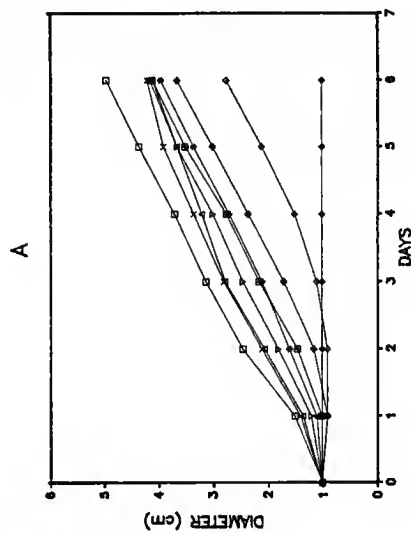
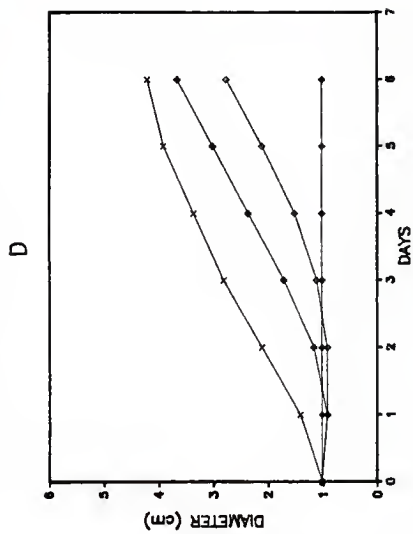
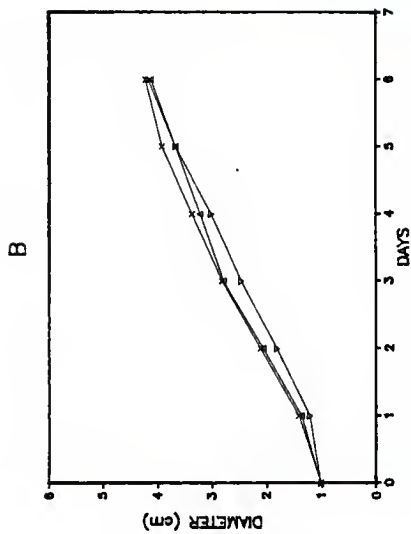
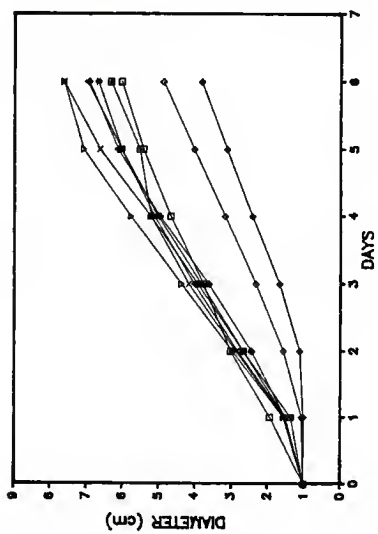


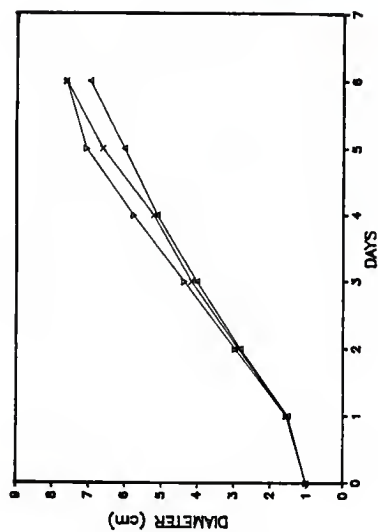
Fig. 33. Diameter of mycelial growth vs. days of culture for A. flavus against SC0719 bound extract and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 5.0% EtOH
—+—	BPDA plus 5.0% EtOH plus tartaric acid (same pH as —◆— )
—▽—	BPDA plus 2.33 mg CE/ml Extract
—▣—	BPDA plus 9.30 mg CE/ml Extract
—◆—	BPDA plus 23.3 mg CE/ml Extract

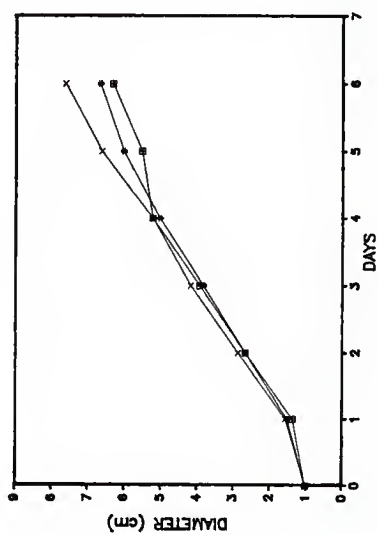
A



B



C



D

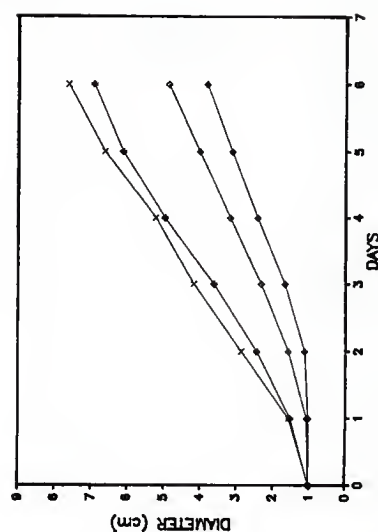


Fig. 34. Diameter of mycelial growth vs. days of culture for F. moniliforme against CS3541 free extract and controls.

<del>—□—</del>	PDA
<del>—X—</del>	BPDA (Buffered PDA)
<del>—△—</del>	BPDA plus 0.3% EtOH
<del>—#—</del>	BPDA plus 1.5% EtOH
<del>—◇—</del>	BPDA plus 3.0% EtOH
<del>—+—</del>	BPDA plus 0.02 mg CE/ml Extract
<del>—▽—</del>	BPDA plus 0.11 mg CE/ml Extract
<del>—⊞—</del>	BPDA plus 0.22 mg CE/ml Extract

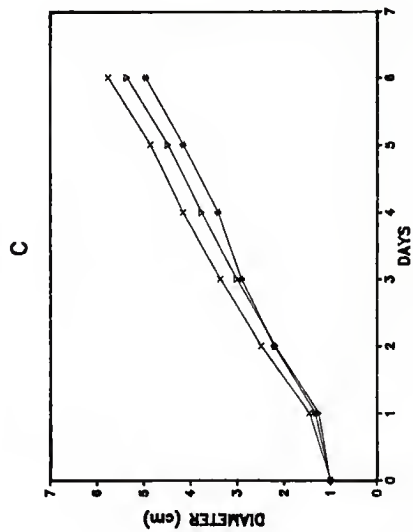
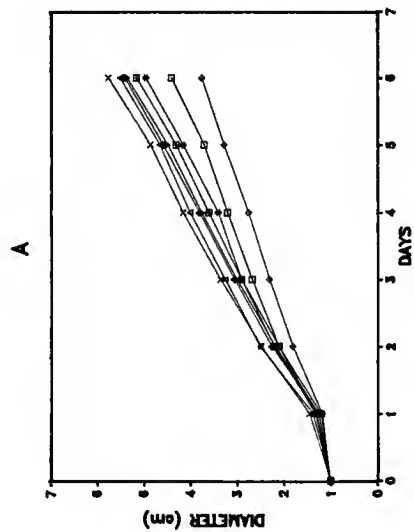
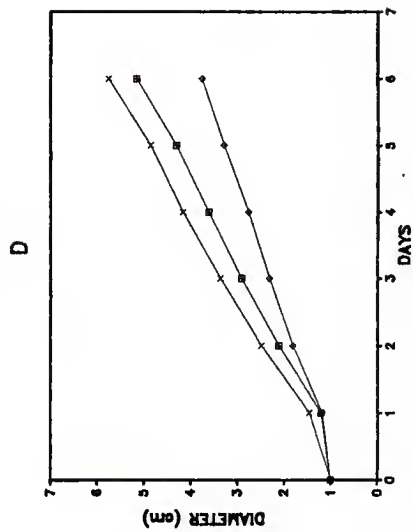
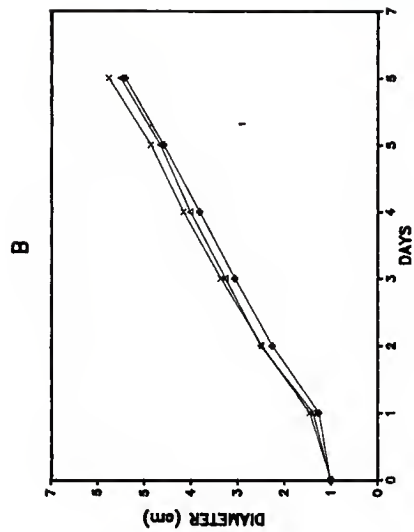
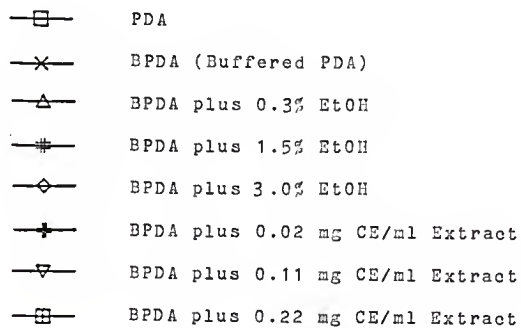


Fig. 35. Diameter of mycelial growth vs. days of culture for *F. semitectum* against CS3541 free extract and controls.



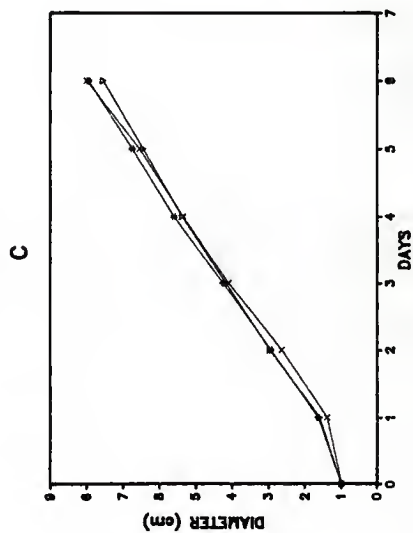
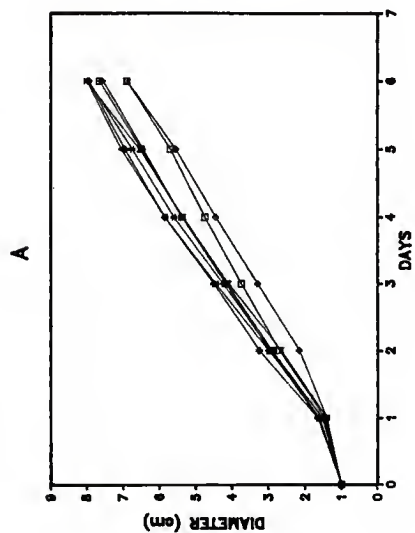
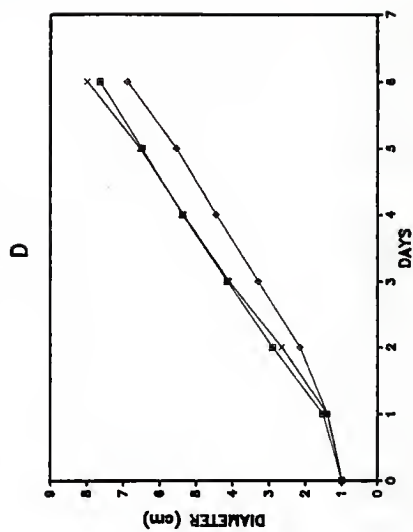
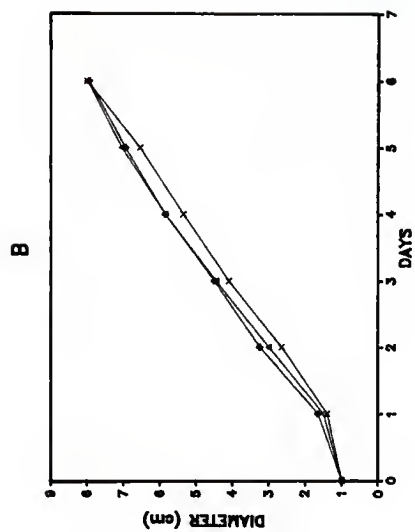


Fig. 36. Diameter of mycelial growth vs. days of culture for F. equiseti against CS3541 free extract and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.3% EtOH
—#—	BPDA plus 1.5% EtOH
—◇—	BPDA plus 3.0% EtOH
—+—	BPDA plus 0.02 mg CE/ml Extract
—▽—	BPDA plus 0.11 mg CE/ml Extract
—⊠—	BPDA plus 0.22 mg CE/ml Extract

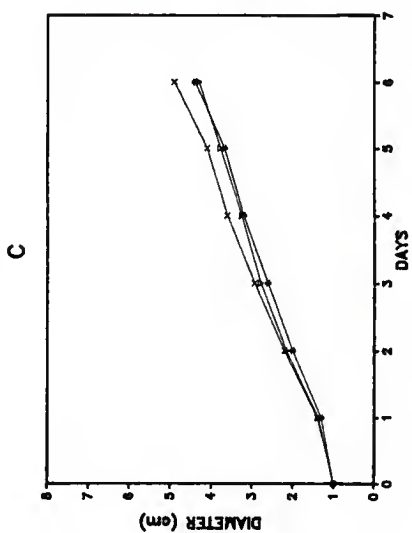
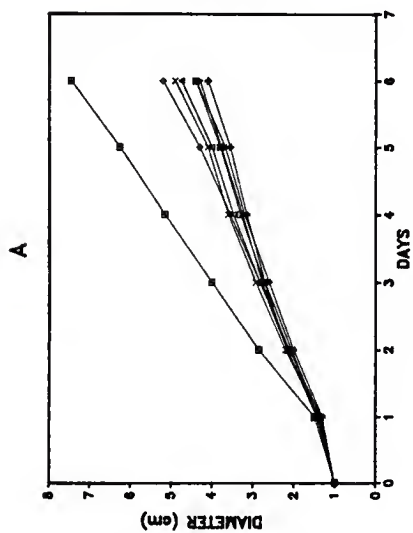
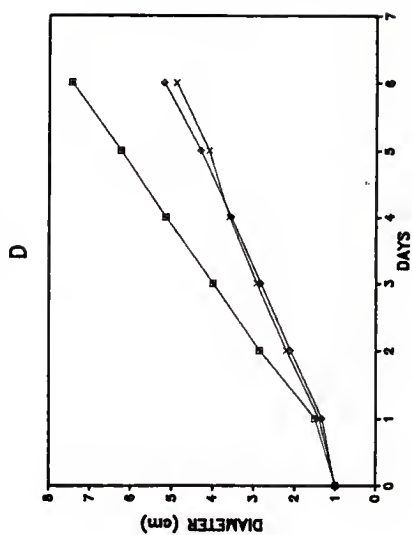
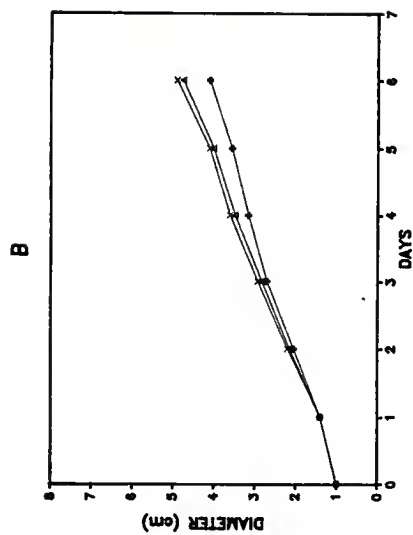








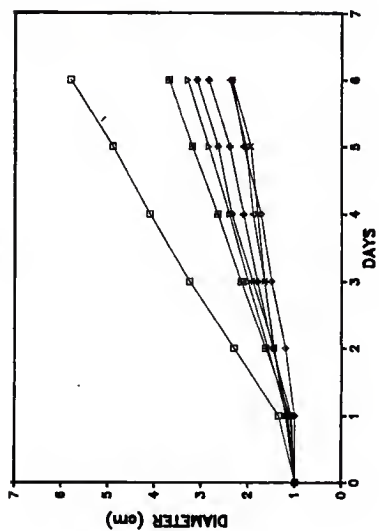


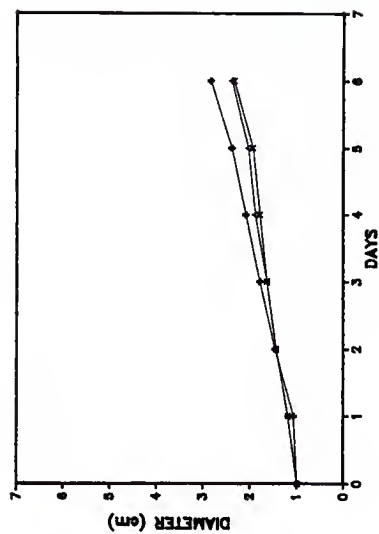
Fig. 37. Diameter of mycelial growth vs. days of culture for A. alternata against CS3541 free extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.3% EtOH
	BPDA plus 1.5% EtOH
	BPDA plus 3.0% EtOH
	BPDA plus 0.02 mg CE/ml Extract
	BPDA plus 0.11 mg CE/ml Extract
	BPDA plus 0.22 mg CE/ml Extract

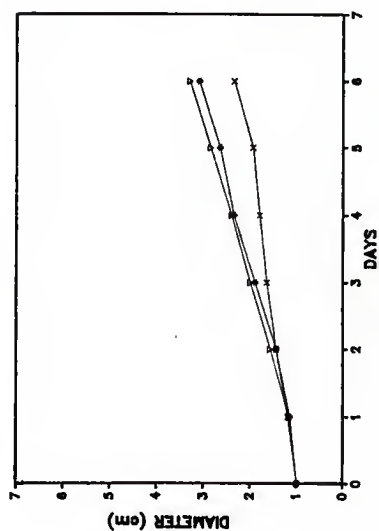
A



B



C



D

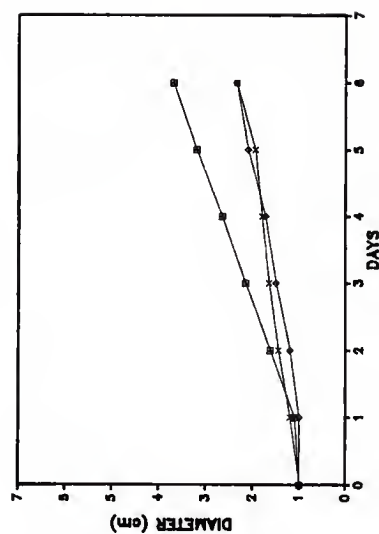








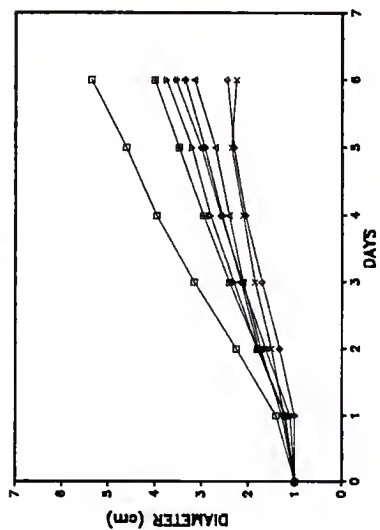


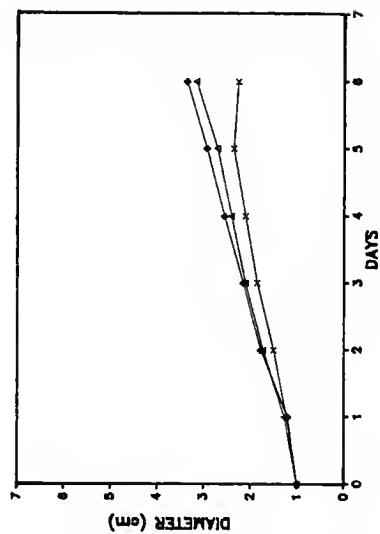
Fig. 38. Diameter of mycelial growth vs. days of culture for A. tenuissima cpx. against CS3541 free extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.3% EtOH
	BPDA plus 1.5% EtOH
	BPDA plus 3.0% EtOH
	BPDA plus 0.02 mg CE/ml Extract
	BPDA plus 0.11 mg CE/ml Extract
	BPDA plus 0.22 mg CE/ml Extract

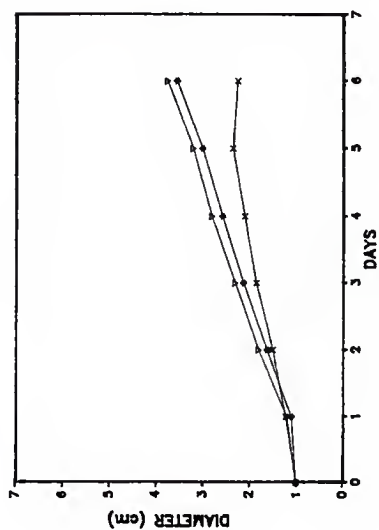
A



B



C



D

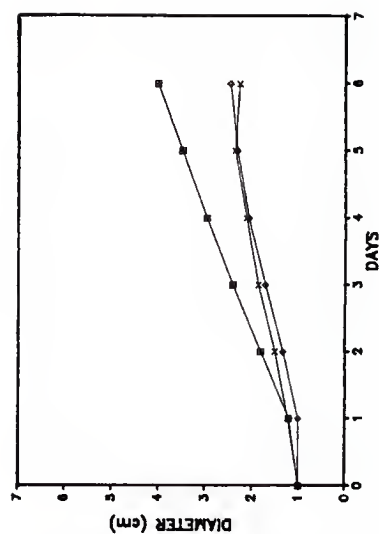










Fig. 39. Diameter of mycelial growth vs. days of culture for A. flavus against CS3541 free extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.3% EtOH
	BPDA plus 1.5% EtOH
	BPDA plus 3.0% EtOH
	BPDA plus 0.02 mg CE/ml Extract
	BPDA plus 0.11 mg CE/ml Extract
	BPDA plus 0.22 mg CE/ml Extract

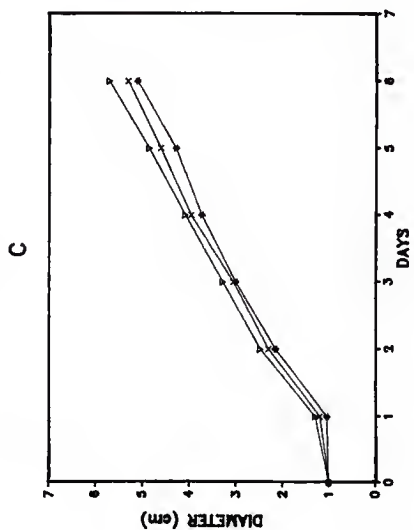
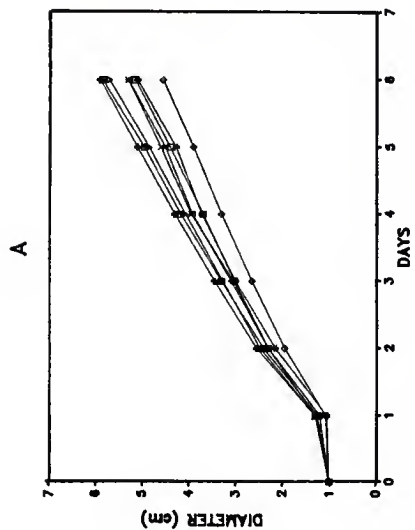
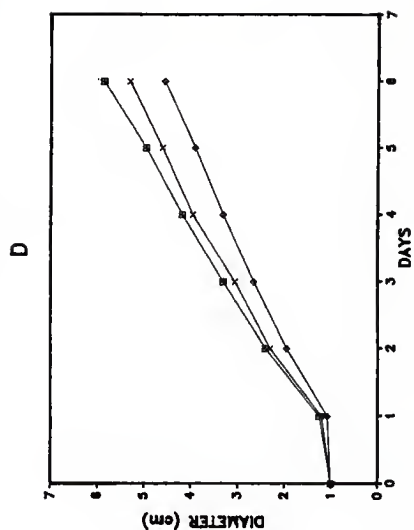
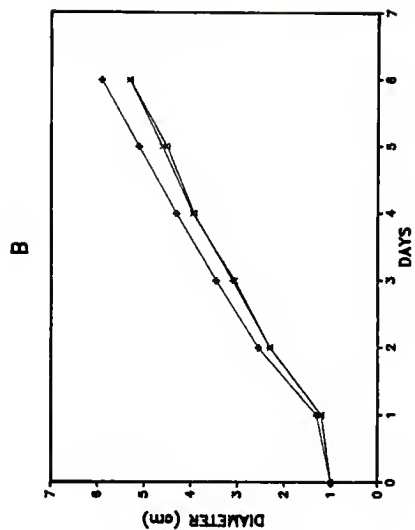
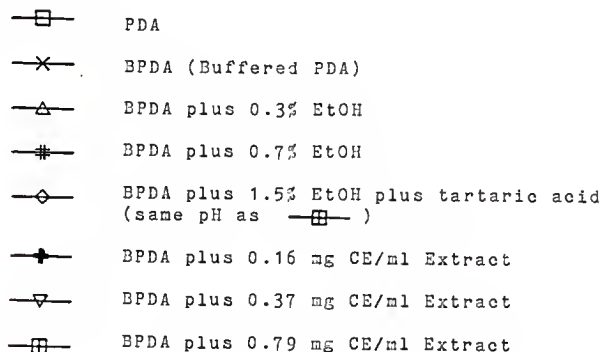
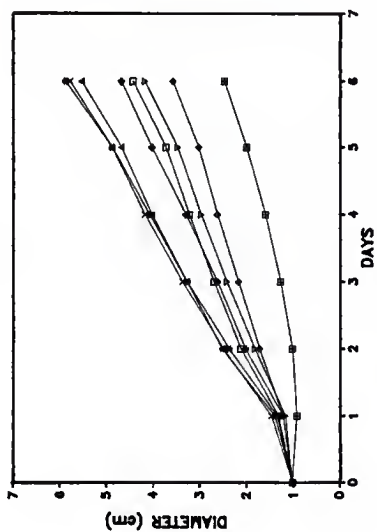


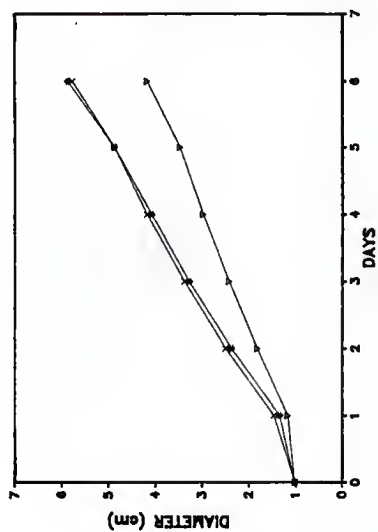
Fig. 40. Diameter of mycelial growth vs. days of culture for E. moniliforme against CS3541 bound extract and controls.



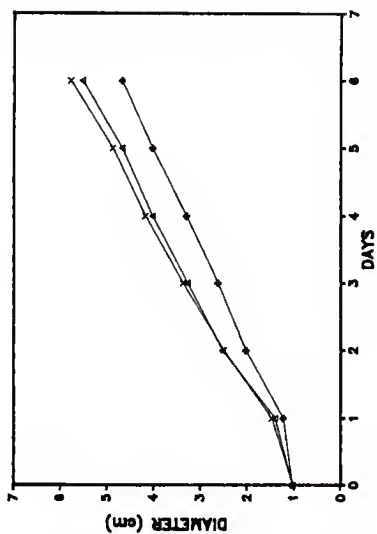
A



C



B



D

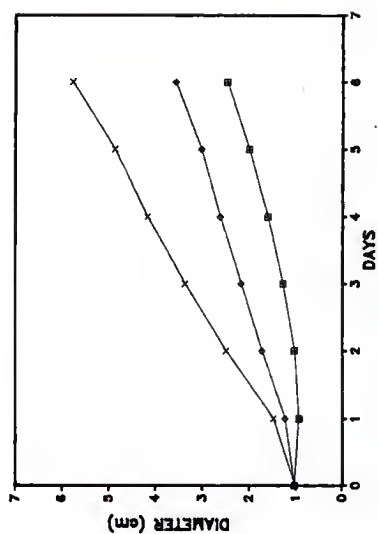











Fig. 41. Diameter of mycelial growth vs. days of culture for F. semitectum against CS3541 bound extract and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.3% EtOH
	BPDA plus 0.7% EtOH
	BPDA plus 1.5% EtOH plus tartaric acid (same pH as  )
	BPDA plus 0.16 mg CE/ml Extract
	BPDA plus 0.37 mg CE/ml Extract
	BPDA plus 0.79 mg CE/ml Extract

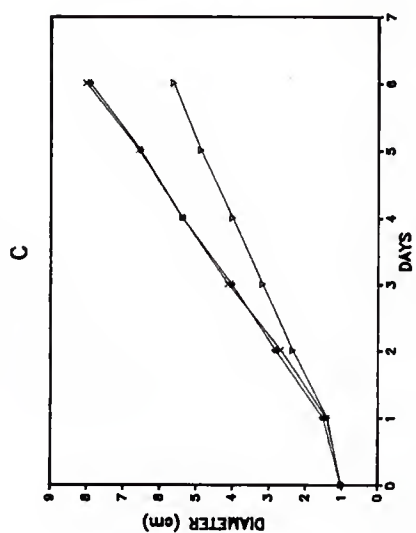
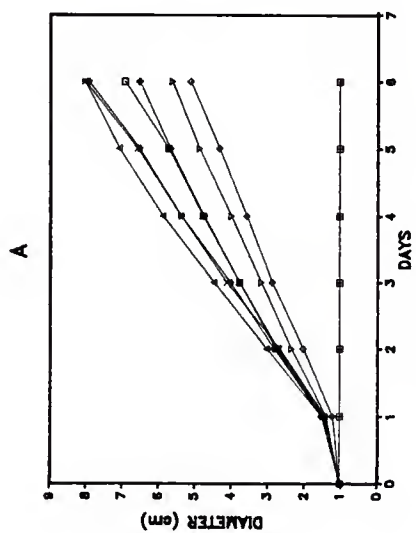
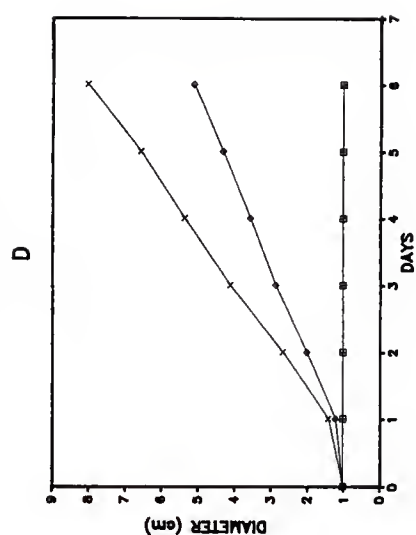
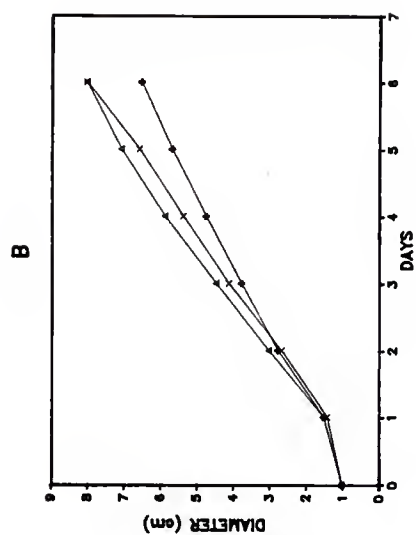
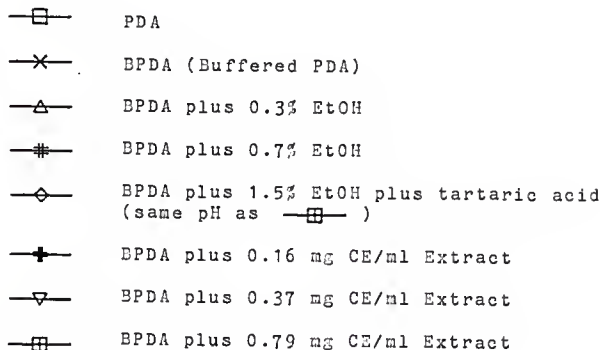


Fig. 42. Diameter of mycelial growth vs. days of culture for F. equiseti against CS3541 bound extract and controls.



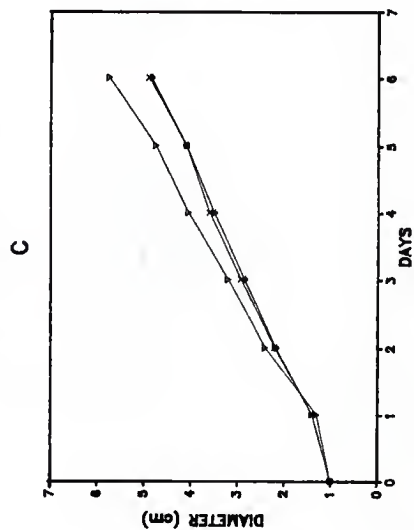
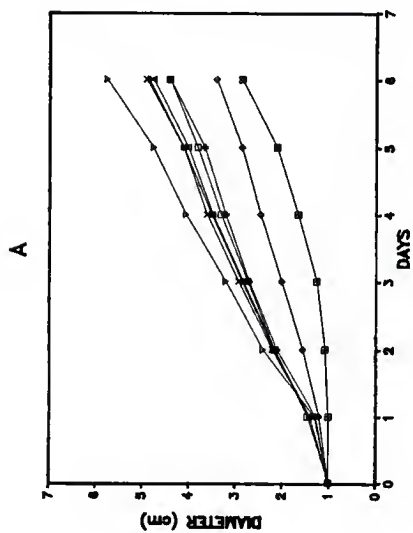
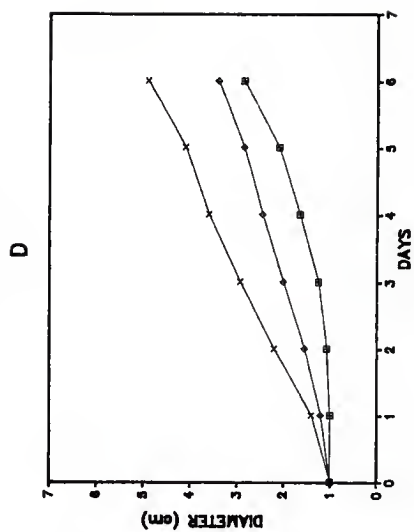
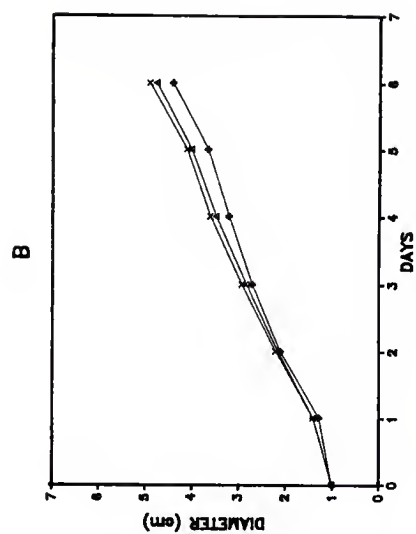
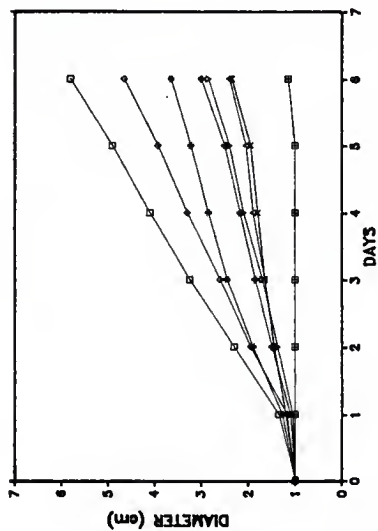


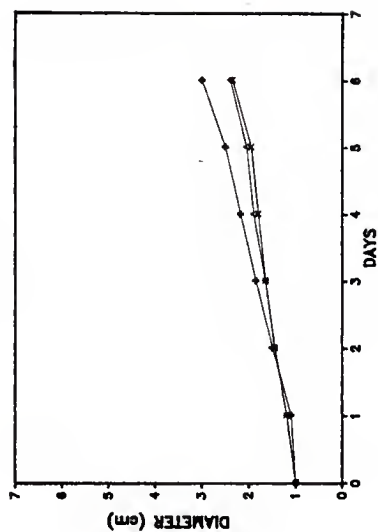
Fig. 43. Diameter of mycelial growth vs. days of culture for A.  
alternata against CS3541 bound extract and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.3% EtOH
—#—	BPDA plus 0.7% EtOH
—◇—	BPDA plus 1.5% EtOH plus tartaric acid (same pH as —▣— )
—+—	BPDA plus 0.16 mg CE/ml Extract
—▽—	BPDA plus 0.37 mg CE/ml Extract
—▣—	BPDA plus 0.79 mg CE/ml Extract

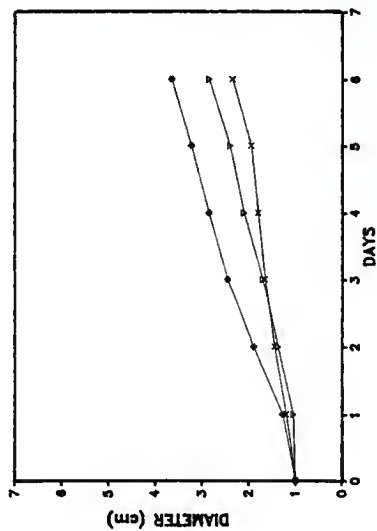
A



B



C



D

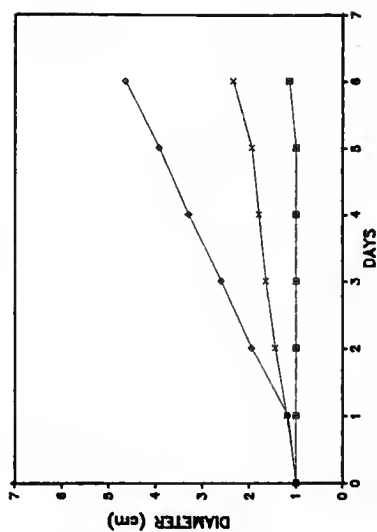
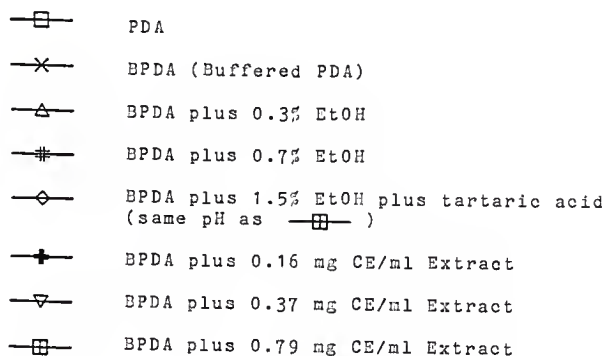
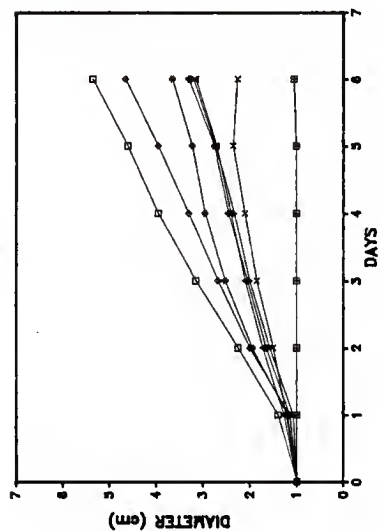


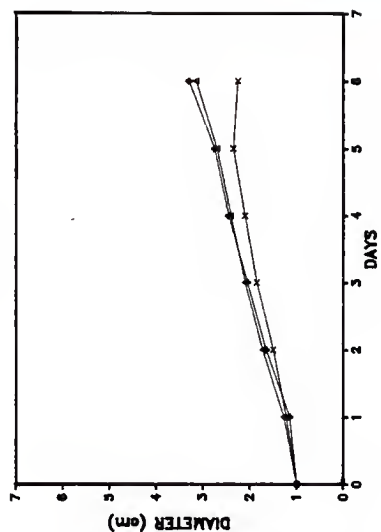
Fig. 44. Diameter of mycelial growth vs. days of culture for A. tenuissima cpx. against CS3541 bound extract and controls.



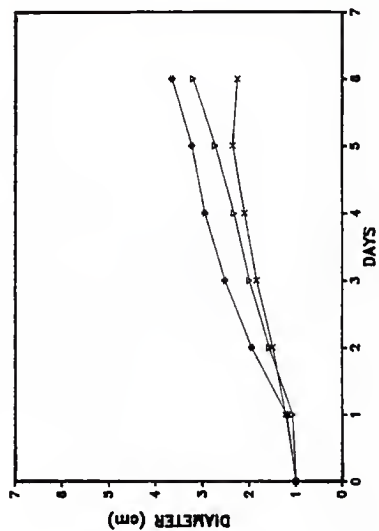
A



B



C



D

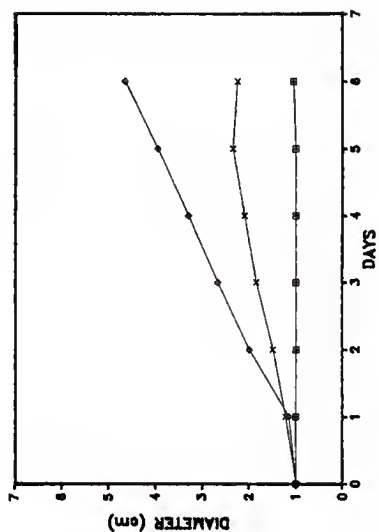
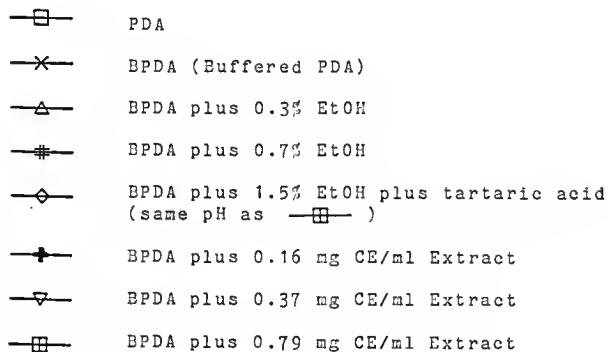
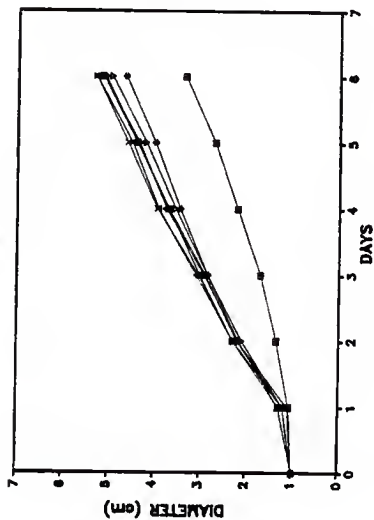


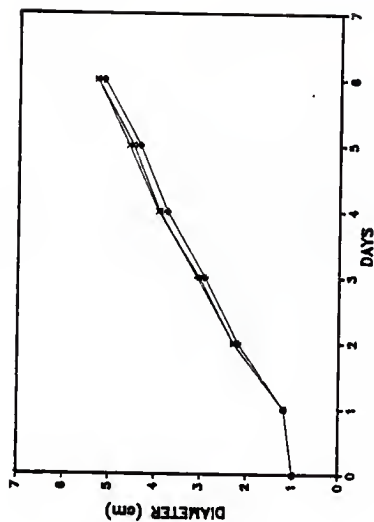
Fig. 45. Diameter of mycelial growth vs. days of culture for A. flavus against CS3541 bound extract and controls.



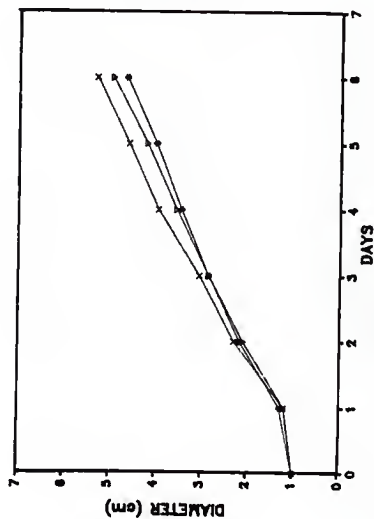
A



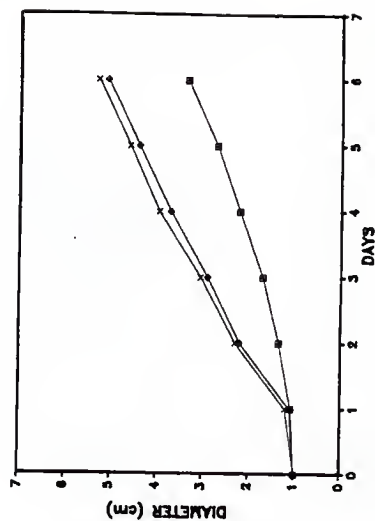
B



C



D



### Activity of model phenolic acids

Ferulic, p-coumaric, vanillic, and cinnamic acids were used for in vitro analyses. Table G, H, I, and J (Appendix) show inhibitory effects. At day 6, of all three phenolic acids had effects on all six fungal isolates. All PDA plates for the in vitro test of model phenolic acids were prepared with 0.2 M phosphate buffer solution, pH 6.4. Amounts of 80% EtOH are equal to that used to dissolve the model phenolic acids. The final concentrations of model phenolic acid solutions were 0.5, 2.0, and 3.5 % (v/v).

The effect of p-coumaric acid against the six fungi is presented graphically in Figs. 46-51. Growth inhibition of E. moniliforme occurred by day 3 at 4mM (Fig. 46C) and inhibition continued to day 6. The high concentration of p-coumaric acid did not show any activity against E. moniliforme (Fig. 46D). This might be due to the effect of high level of 80% EtOH on E. moniliforme which concealed or overshadowed any inhibition due to the phenolic acid. No inhibition (Figs. 46B) was shown at low concentration. p-Coumaric acid inhibited growth of E. semitectum (Fig. 47) and E. equiseti (Fig. 48) at concentrations down to 1mM and the inhibitions at all three concentrations were apparent by day 2 and 1 respectively. At high concentration, p-coumaric acid appeared to have the same phenomenon against E. moniliforme (probably the effect of 80% EtOH). Growth inhibition of A. alternata (Fig. 49) was detected by day 3 at a 7 mM minimum. Whereas, the growth inhibition of A. tenuissima cpx. (Fig. 50) started by day 1 at 1 mM. In the case of A. flavus (fig. 51), no inhibition was observed at any concentration of p-coumaric acid.

Ferulic acid (Table H, Appendix) was a more effective inhibitor of *Fusarium* spp. and *A. flavus* than p-coumaric acid. At 1 mM, growth inhibition was detected by day 2 for *F. moniliforme* (Fig. 52) and *F. semitectum* (Fig. 53), and by day 1 for *F. equiseti* (fig. 54). Ferulic acid inhibited growth of *A. flavus* (Fig. 57) by day 4 at a 4 mM minimum. For *A. alternata* (Fig. 55), only slight growth inhibition was observed by day 5 at 1 mM. No inhibition was apparent at higher concentrations. However, ferulic acid inhibited growth of *A. tenuissima* cpx. (Fig. 56) by day 1 at 1mM.

Vanillic acid (Table I, Appendix) was the least effective phenolic acid among the four acids tested. There was no inhibition observed for *A. alternata* (Fig. 61), *A. tenuissima* cpx. (Fig. 62), and *A. flavus* (Fig. 63). Minor growth inhibition was observed at 1 mM for *F. moniliforme* (Fig. 58). In the case of *F. semitectum* (Fig. 59), growth inhibition occurred by day 2 at 7 mM. However, vanillic acid was most effective against *F. equiseti* (Fig. 60) inhibiting growth as early as day 1 at 1 mM.

Cinnamic acid (Table J, Appendix) was the most effective phenolic acid tested. The effect of cinnamic acid on all six selected fungi (Figs. 64-69) was detected as early as day 1 or day 2 at 1 mM minimum concentration. For *A. alternata* (Fig. 67) and *A. tenuissima* cpx. (Fig. 68), growth was inhibited almost completely at 4 mM and completely at 7 mM.

All four purified phenolic acids chosen as model compounds exist naturally in sorghum samples (Table 9). All were effective in inhibiting fungal growth *in vitro* when included in growth medium after autoclaving and prior to plating. The only exceptions were vanillic acid against *A. alternata*, *A. tenuissima* cpx., and *A.*

flavus, and p-coumaric acid against A. flavus. Table 3 shows the minimum inhibitory concentrations against fungal species.

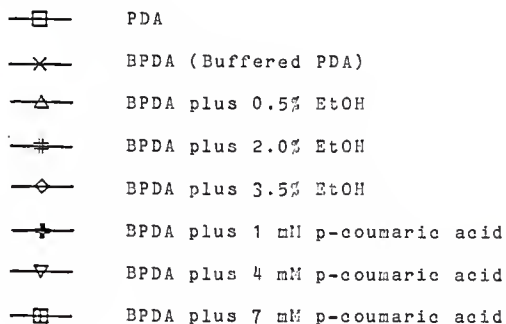
In order of decreasing antifungal activity the four model phenolic acids, were cinnamic, ferulic, p-coumaric, and vanillic acid. Decreasing polarity of phenolic acid corresponded with increases in inhibitory activity. Similar observations with flavonoids were made by O'Neill and Mansfield (1982), and Ingham (1977). O'Neill and Mansfield (1982) reported that the presence of one or two polar groups in hydroxyflavans and other flavonoids, but not more, appears to be necessary for activity while complete methylation of hydroxyl groups removes activity. Perrin and Cruickshank (1969) proposed that a common molecular shape was responsible for activity.

TABLE 3

Minimum inhibitory concentrations vs fungal species

-----	
CINNAMIC	FERULIC
<i>F. <u>equiseti</u></i> (1mM)	<i>F. <u>equiseti</u></i> (1mM)
<i>F. <u>moniliforme</u></i> (1mM)	<i>F. <u>moniliforme</u></i> (1mM)
<i>F. <u>semitectum</u></i> (1mM)	<i>F. <u>semitectum</u></i> (1mM)
<i>A. <u>tenuissima</u></i> cpx. (1mM)	<i>A. <u>tenuissima</u></i> cpx. (1mM)
<i>A. <u>alternata</u></i> (1mM)	<i>A. <u>alternata</u></i> (1mM)
<i>A. <u>flavus</u></i> (1mM)	<i>A. <u>flavus</u></i> (4mM)
P-COUMARIC	VANILLIC
<i>F. <u>equiseti</u></i> (1mM)	<i>F. <u>equiseti</u></i> (1mM)
<i>F. <u>moniliforme</u></i> (4mM)	<i>F. <u>moniliforme</u></i> (1mM)
<i>F. <u>semitectum</u></i> (1mM)	<i>F. <u>semitectum</u></i> (7mM)
<i>A. <u>tenuissima</u></i> cpx. (1mM)	
<i>A. <u>alternata</u></i> (7mM)	
-----	

Fig. 46. Diameter of mycelial growth vs. days of culture for F. moniliforme against p-coumaric acid and controls.



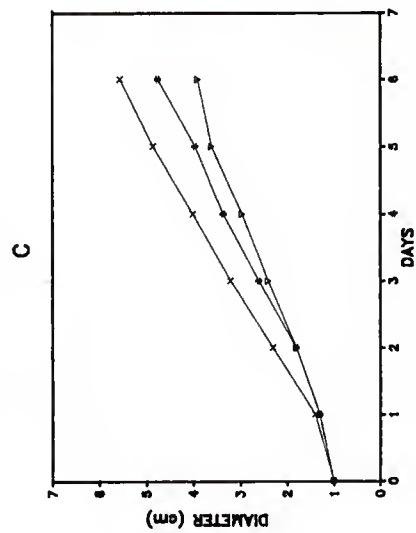
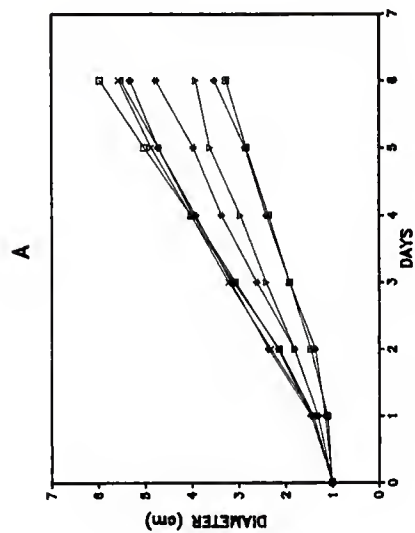
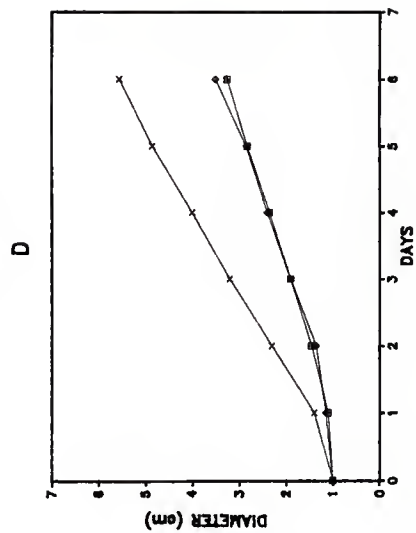
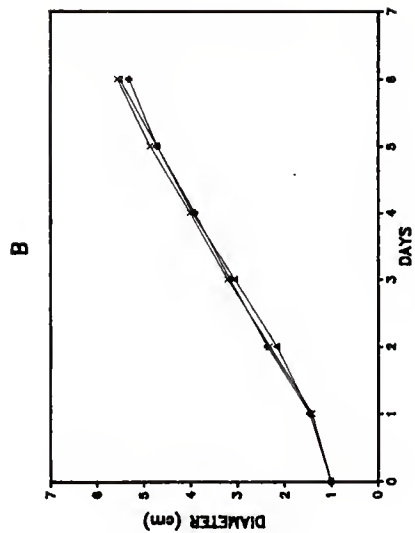


Fig. 47. Diameter of mycelial growth vs. days of culture for F. semitectum against p-coumaric acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM p-coumaric acid
—▽—	BPDA plus 4 mM p-coumaric acid
—■—	BPDA plus 7 mM p-coumaric acid

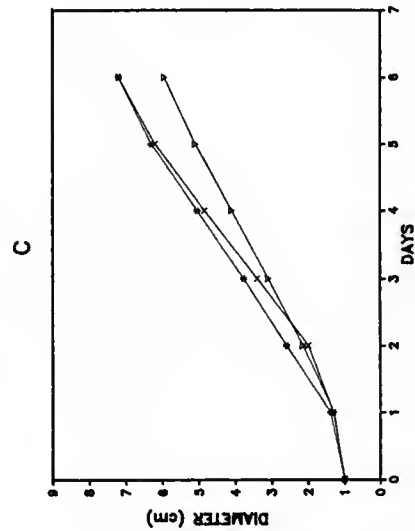
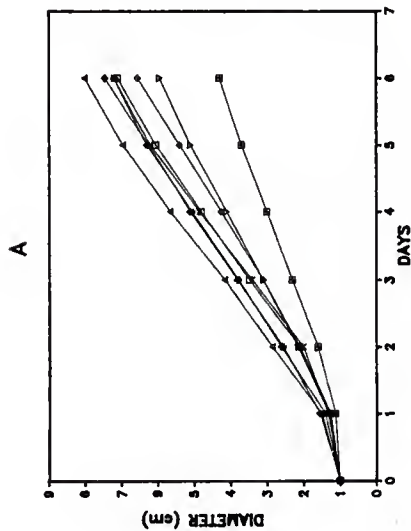
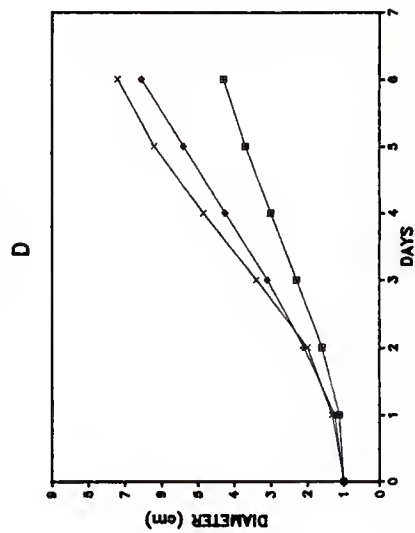
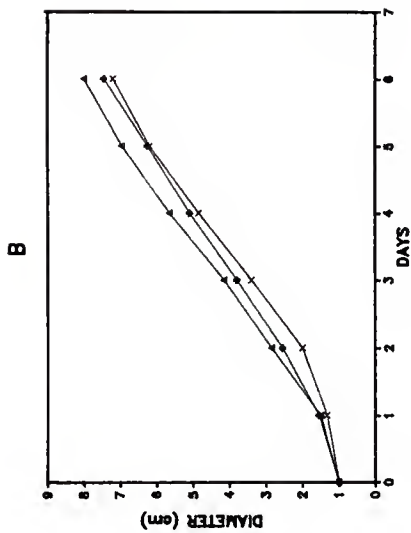


Fig. 48. Diameter of mycelial growth vs. days of culture for F. equiseti against p-coumaric acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—⊞—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—⊕—	BPDA plus 1 mM p-coumaric acid
—▽—	BPDA plus 4 mM p-coumaric acid
—⊠—	BPDA plus 7 mM p-coumaric acid

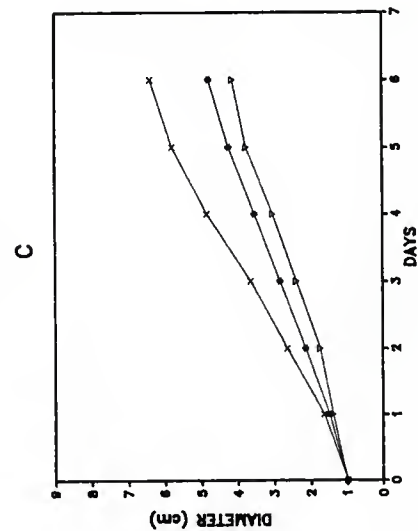
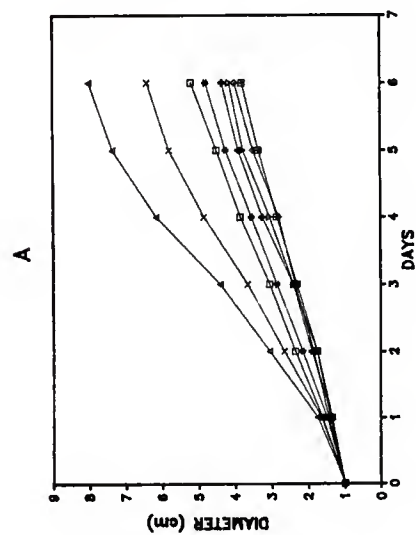
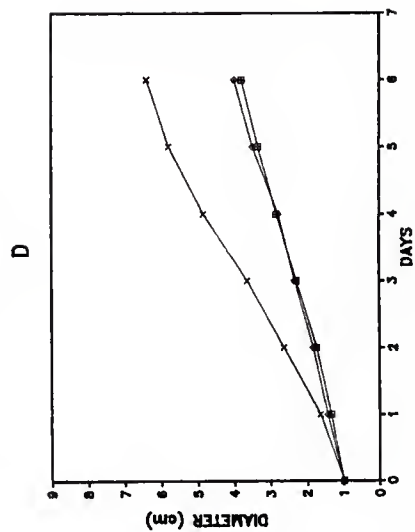
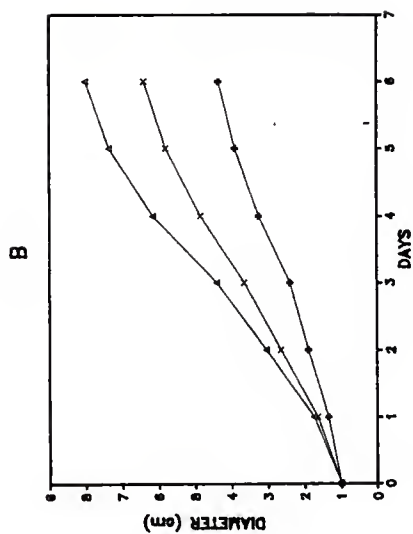
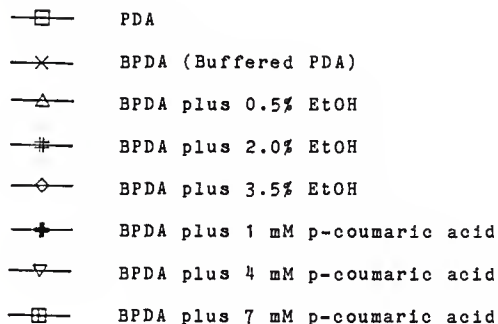


Fig. 49. Diameter of mycelial growth vs. days of culture for  
A. alternata against p-coumaric acid and controls.



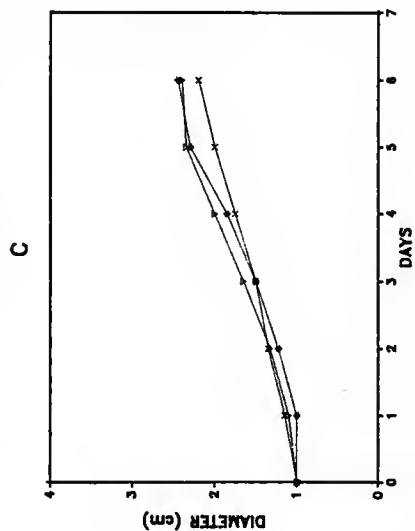
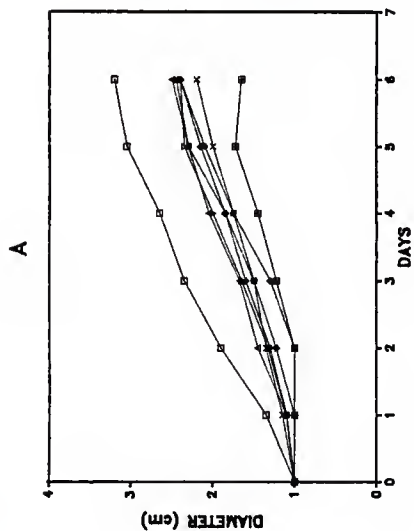
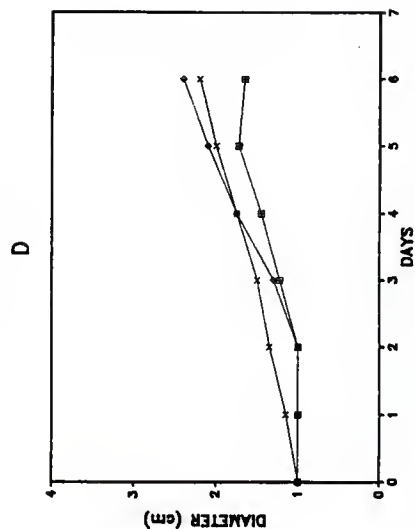
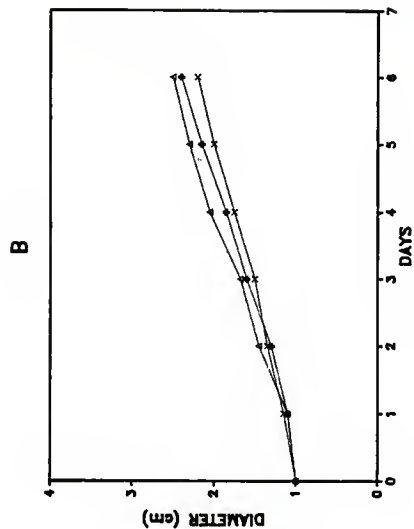


Fig. 50. Diameter of mycelial growth vs. days of culture for A.  
tenuissima cpx. against p-coumaric acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM p-coumaric acid
—▽—	BPDA plus 4 mM p-coumaric acid
—▣—	BPDA plus 7 mM p-coumaric acid

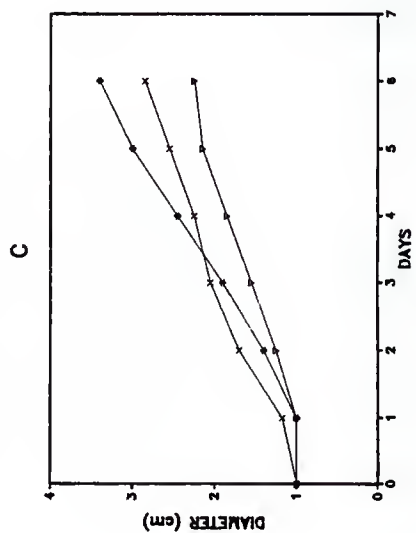
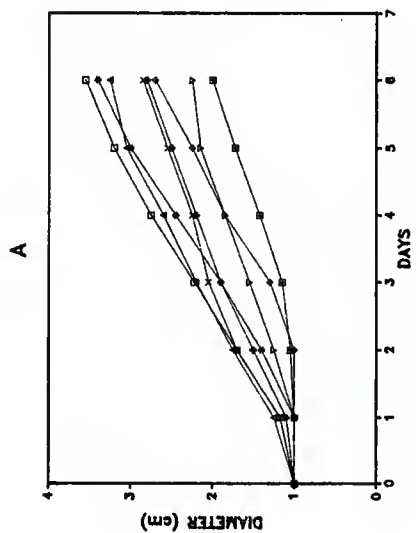
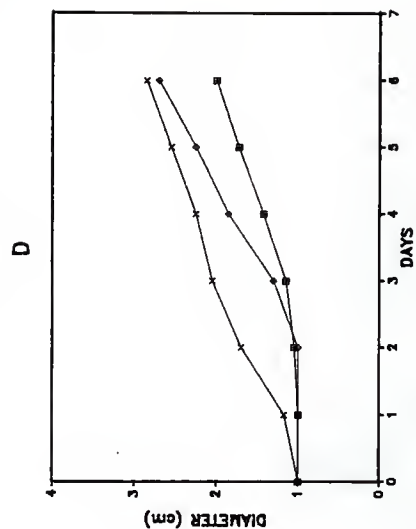
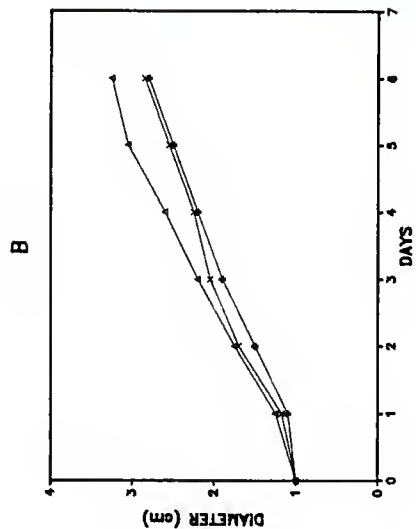


Fig. 51. Diameter of mycelial growth vs. days of culture for A. flavus against p-coumaric acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—≡—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—⊕—	BPDA plus 1 mM p-coumaric acid
—▽—	BPDA plus 4 mM p-coumaric acid
—⊞—	BPDA plus 7 mM p-coumaric acid

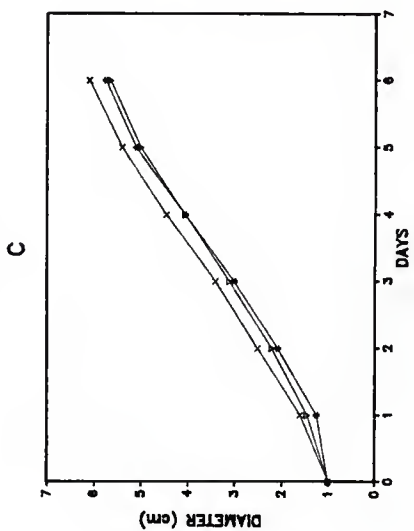
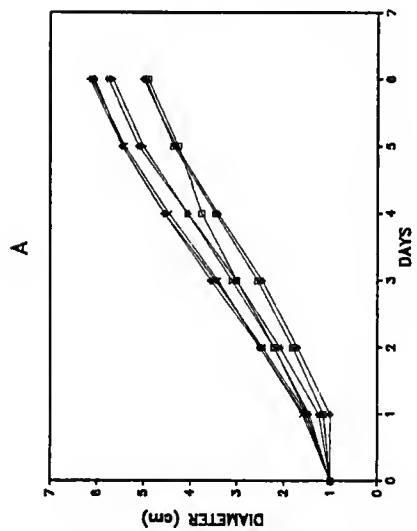
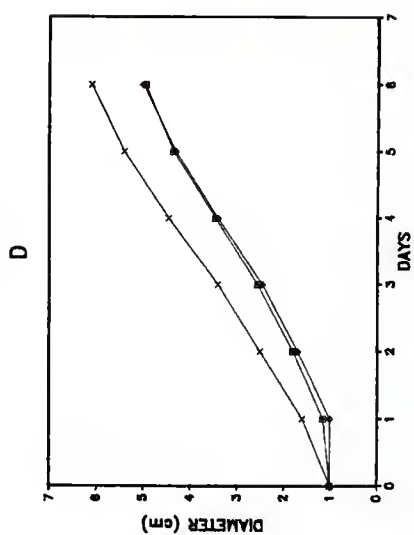
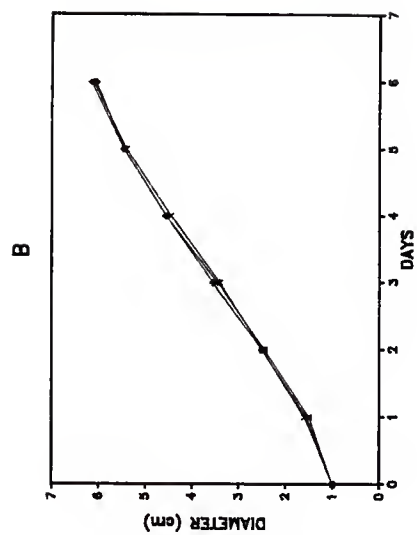


Fig. 52. Diameter of mycelial growth vs. days of culture for F. moniliforme against ferulic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM ferulic acid
—▽—	BPDA plus 4 mM ferulic acid
—▣—	BPDA plus 7 mM ferulic acid

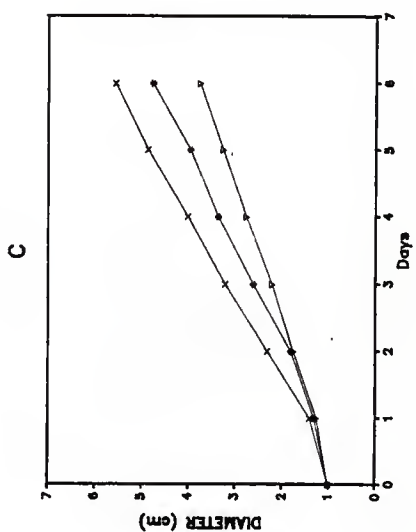
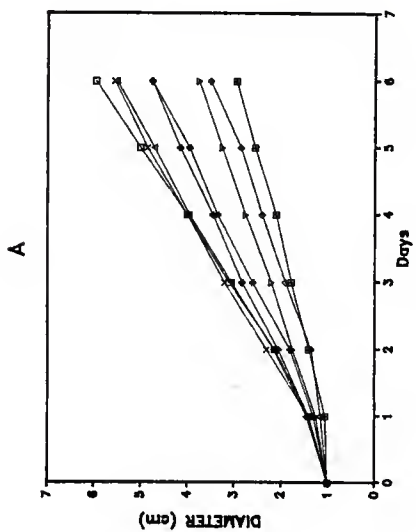
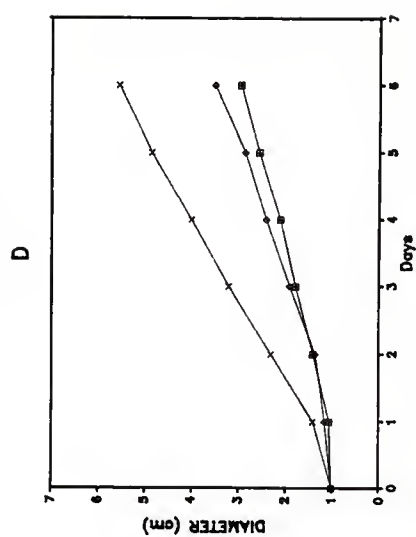
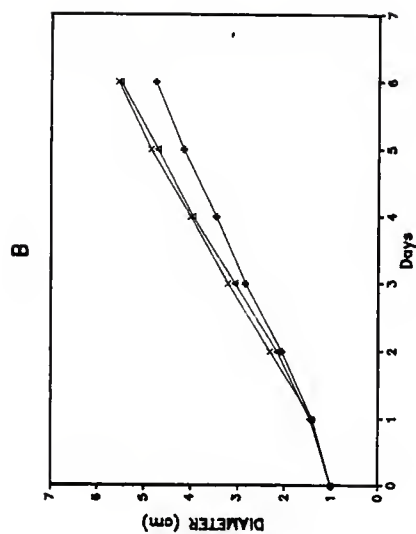
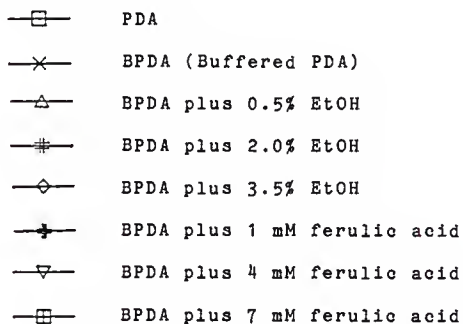
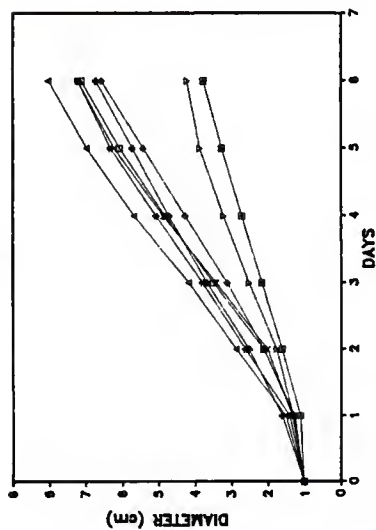


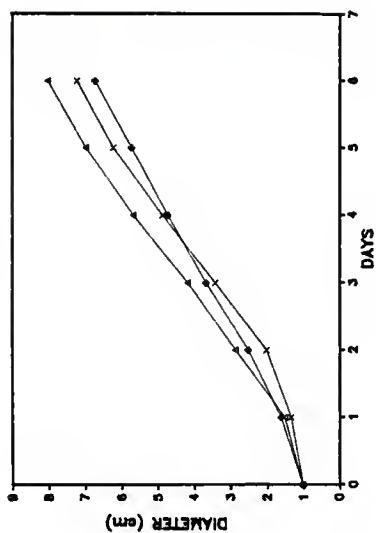
Fig. 53. Diameter of mycelial growth vs. days of culture for F. semitectum against ferulic acid and controls.



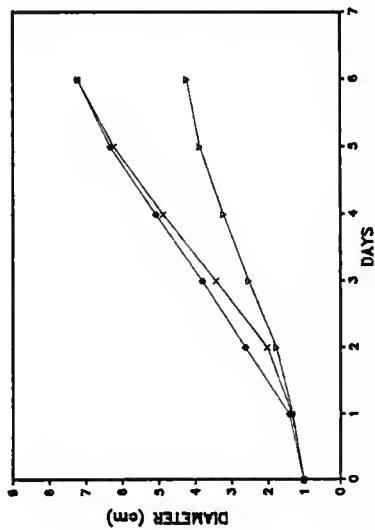
A



B



C



D

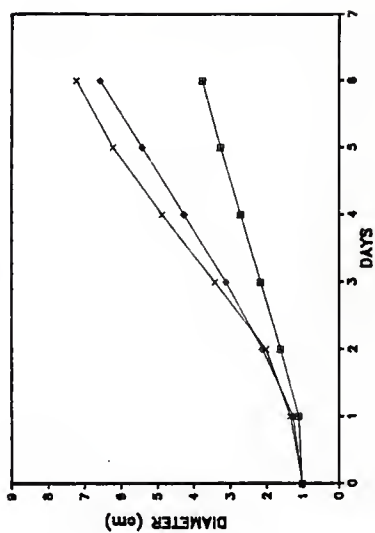
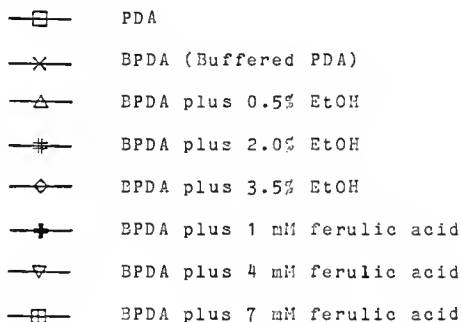


Fig. 54. Diameter of mycelial growth vs. days of culture for F. equiseti against ferulic acid and controls.



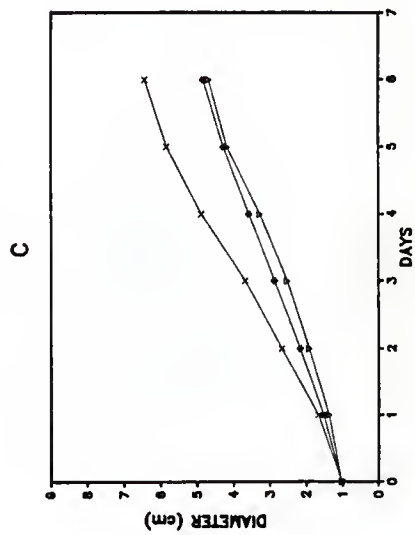
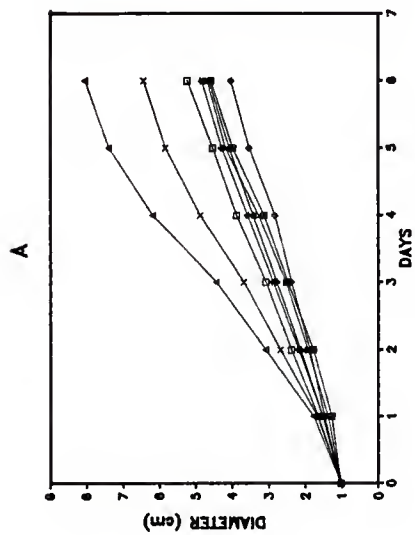
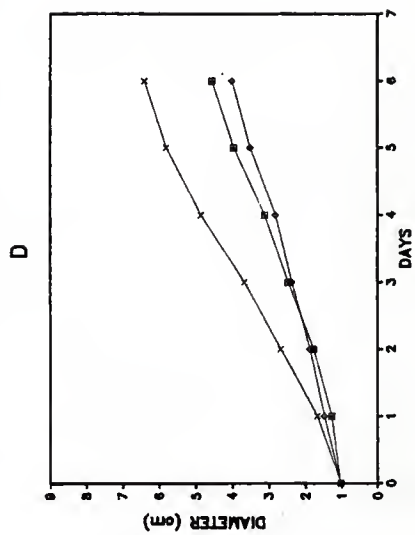
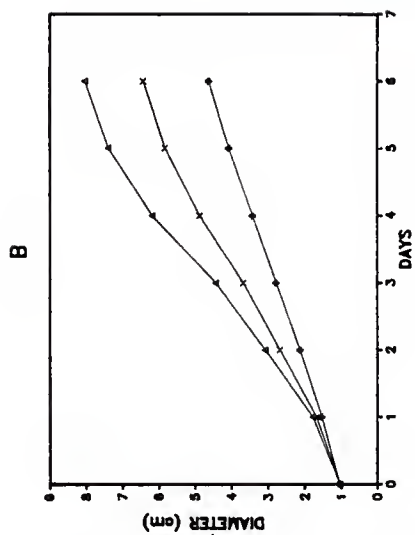
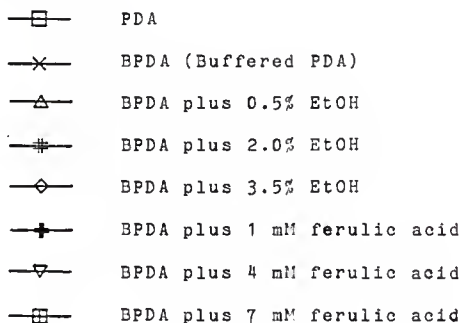
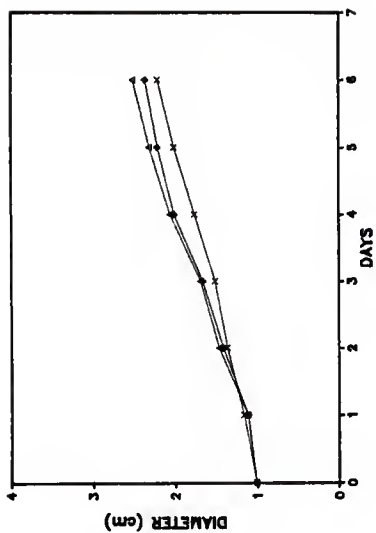


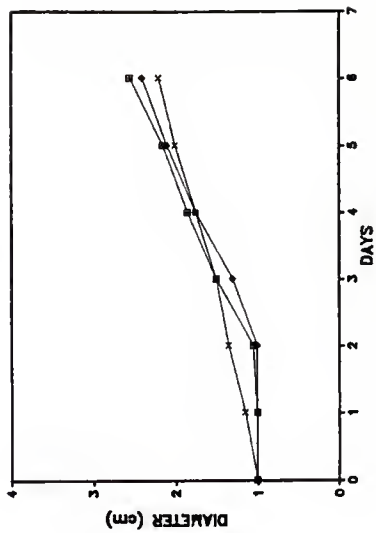
Fig. 55. Diameter of mycelial growth vs. days of culture for  
*A. alternata* against ferulic acid and controls.



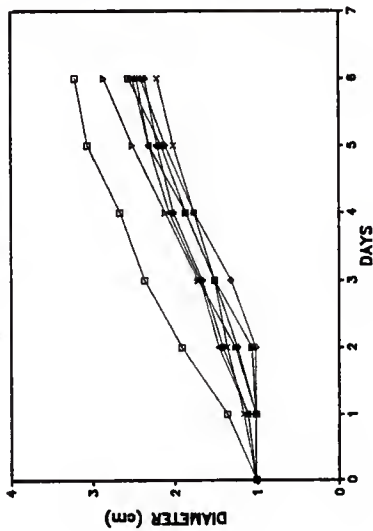
B



D



A



C

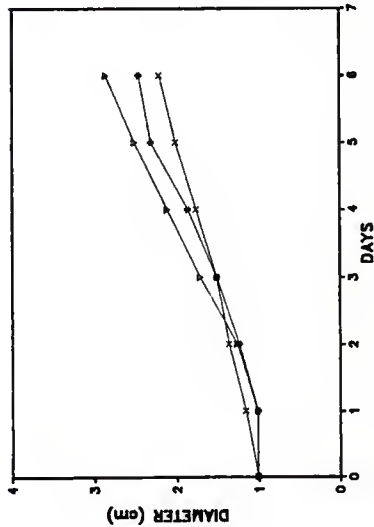


Fig. 56. Diameter of mycelial growth vs. days of culture for A. tenuissima cpx. against ferulic acid and controls.

—□—	PDA
—×—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM ferulic acid
—▽—	BPDA plus 4 mM ferulic acid
—⊠—	BPDA plus 7 mM ferulic acid

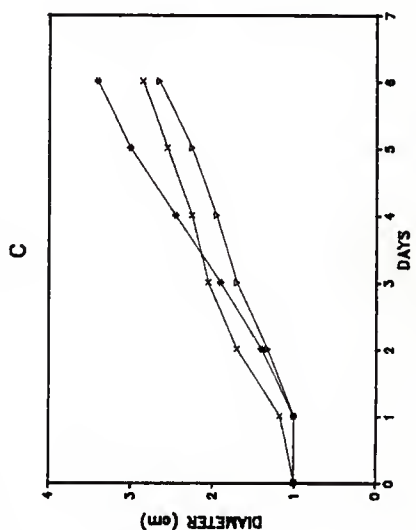
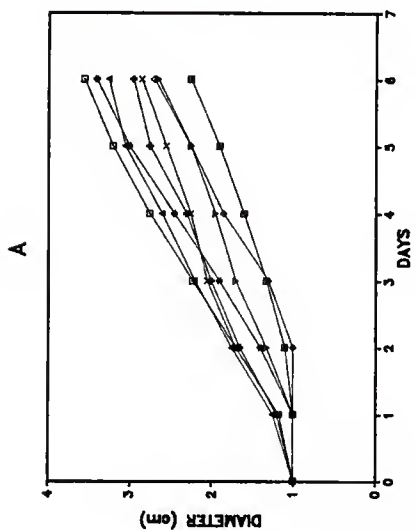
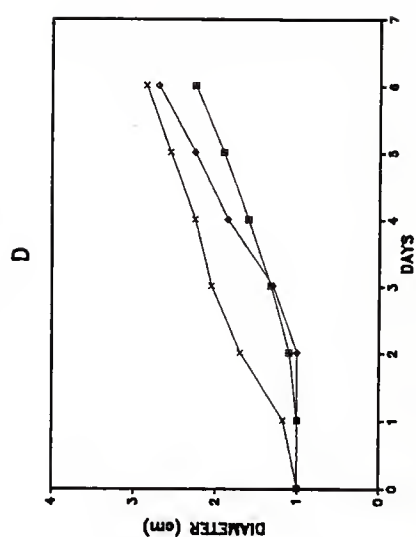
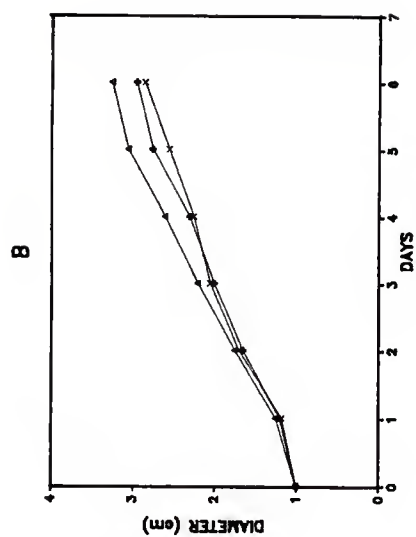
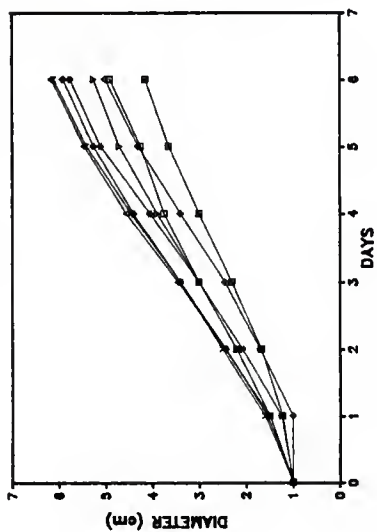


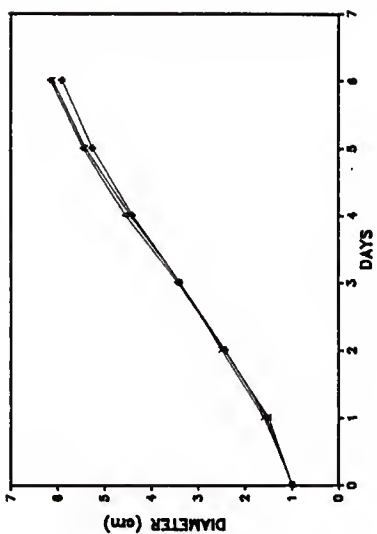
Fig. 57. Diameter of mycelial growth vs. days of culture for A. flavus against ferulic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—⊕—	BPDA plus 1 mM ferulic acid
—▽—	BPDA plus 4 mM ferulic acid
—▣—	BPDA plus 7 mM ferulic acid

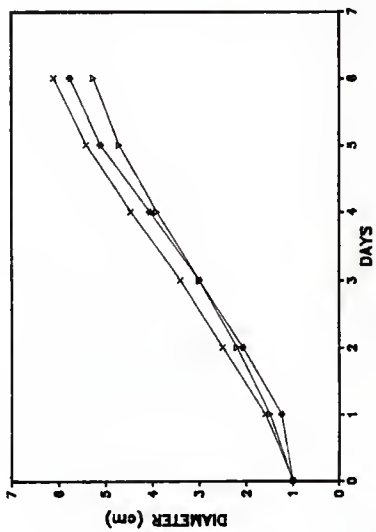
A



B



C



D

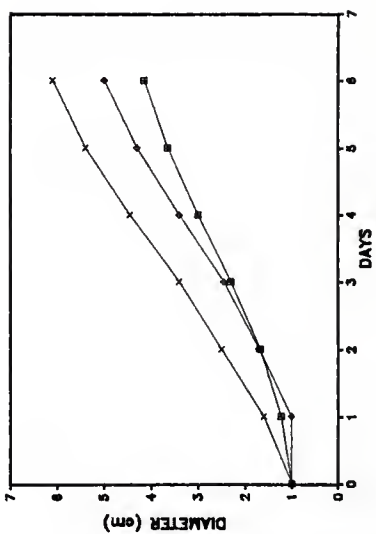
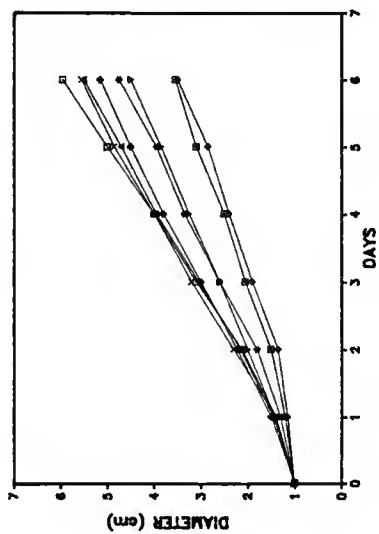


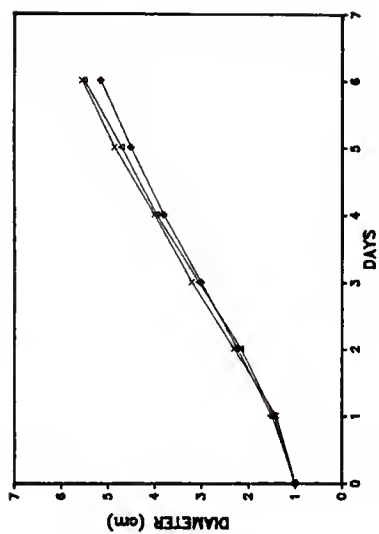
Fig. 58. Diameter of mycelial growth vs. days of culture for F. moniliforme against vanillic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—⊕—	BPDA plus 1 mM vanillic acid
—▽—	BPDA plus 4 mM vanillic acid
—⊞—	BPDA plus 7 mM vanillic acid

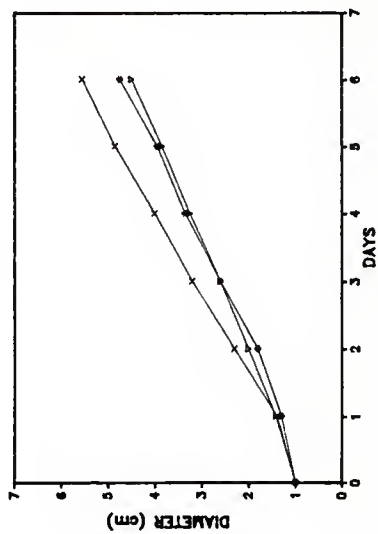
A



B



C



D

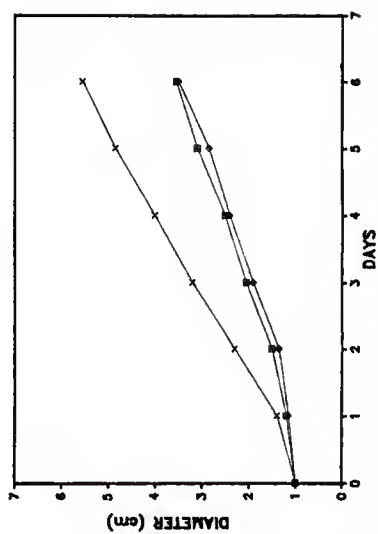
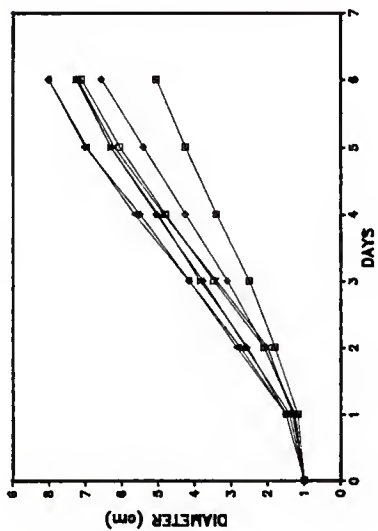


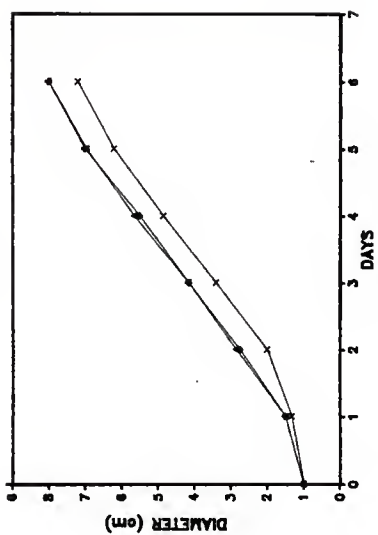
Fig. 59. Diameter of mycelial growth vs. days of culture for E. semitectum against vanillic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM vanillic acid
—▽—	BPDA plus 4 mM vanillic acid
—▣—	BPDA plus 7 mM vanillic acid

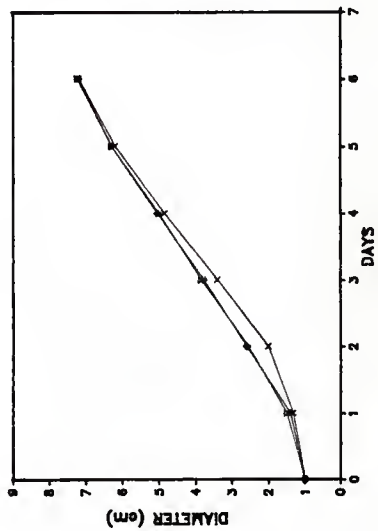
A



B



C



D

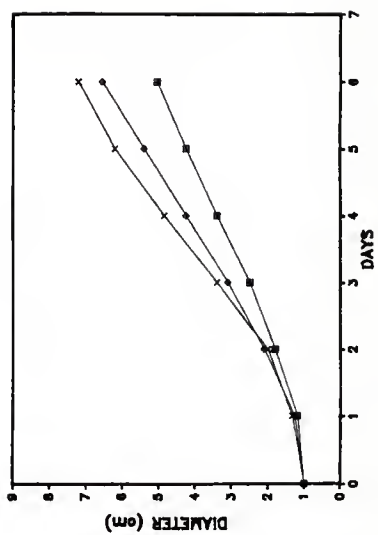
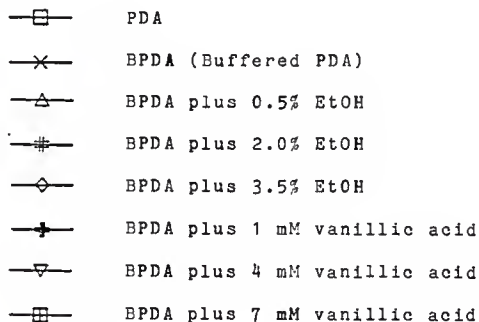


Fig. 60. Diameter of mycelial growth vs. days of culture for F. equiseti against vanillic acid and controls.



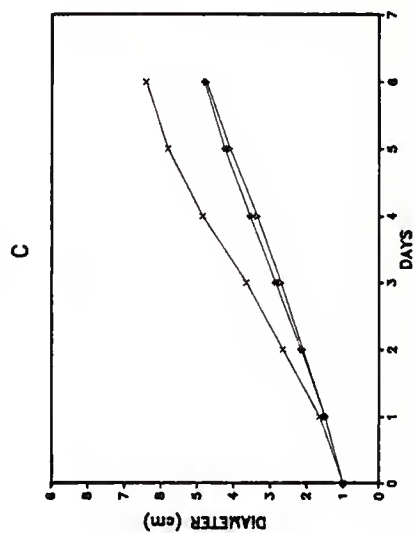
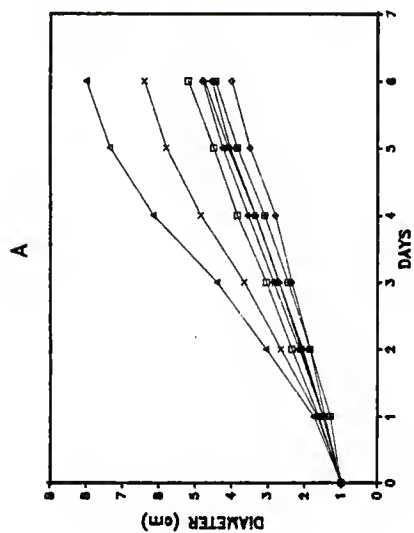
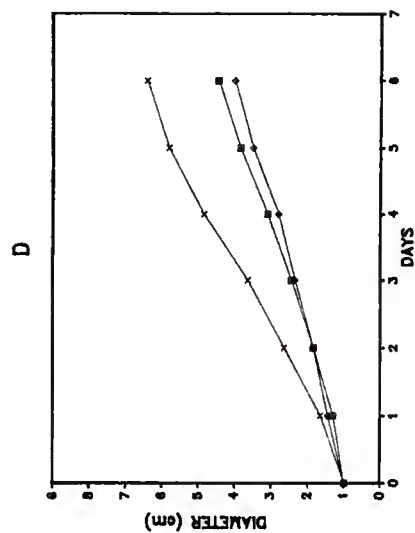
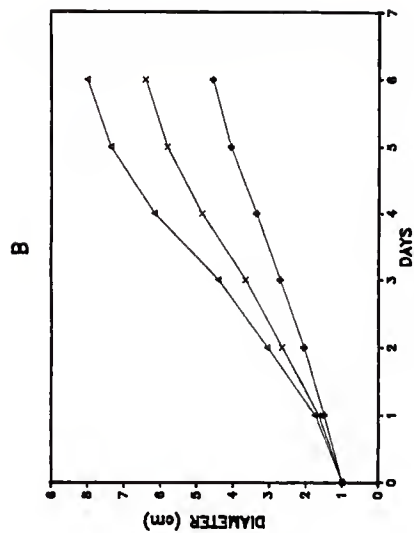
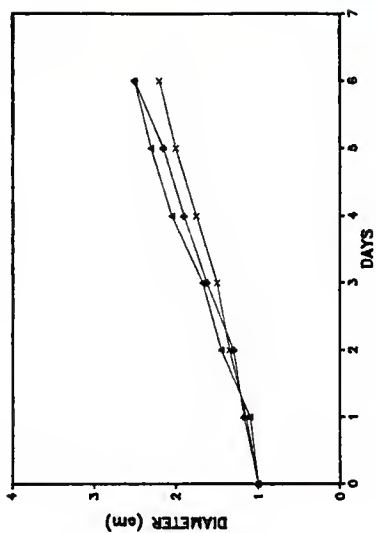


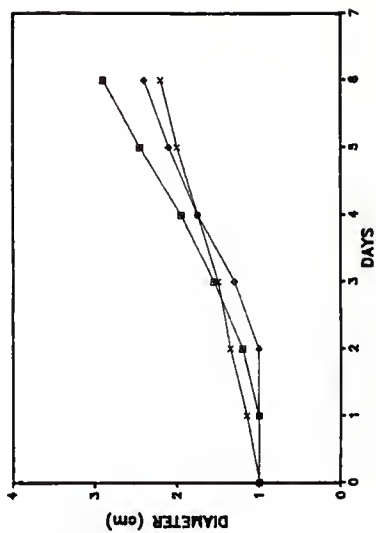
Fig. 61. Diameter of mycelial growth vs. days of culture for A. alternata against vanillic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM vanillic acid
—▽—	BPDA plus 4 mM vanillic acid
—▣—	BPDA plus 7 mM vanillic acid

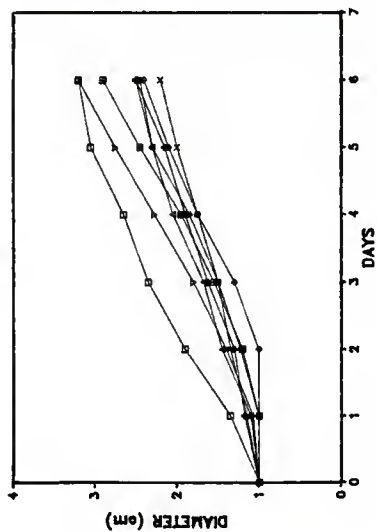
B



D



A



C

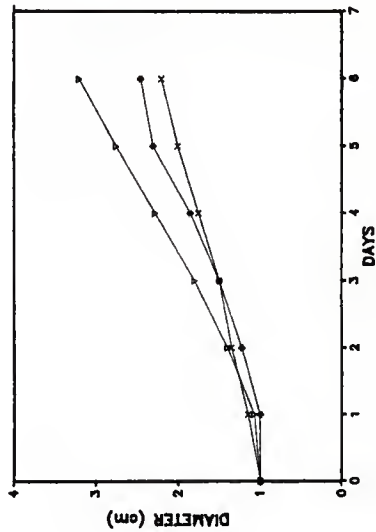










Fig. 62. Diameter of mycelial growth vs. days of culture for *A. tenuissima* cpx. against vanillic acid and controls.

	PDA
	BPDA (Buffered PDA)
	BPDA plus 0.5% EtOH
	BPDA plus 2.0% EtOH
	BPDA plus 3.5% EtOH
	BPDA plus 1 mM vanillic acid
	BPDA plus 4 mM vanillic acid
	BPDA plus 7 mM vanillic acid

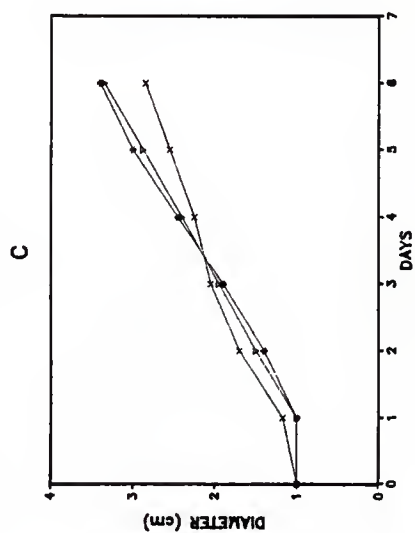
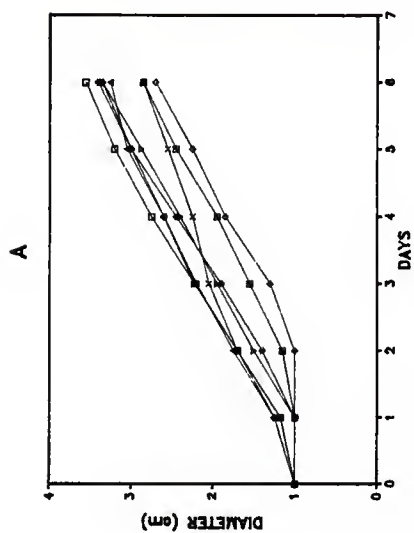
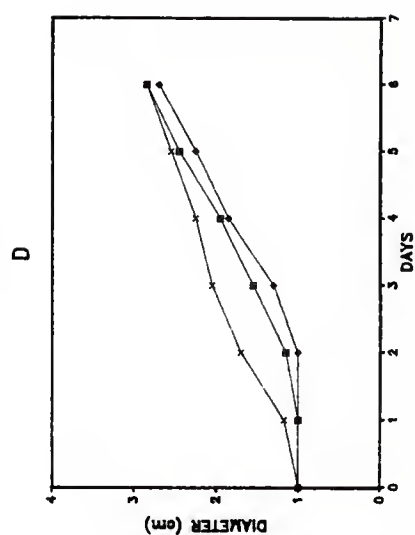
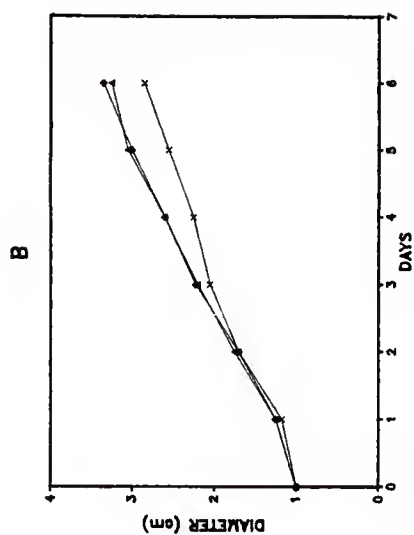


Fig. 63. Diameter of mycelial growth vs. days of culture for  
*A. flavus* against vanillic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—⊕—	BPDA plus 1 mM vanillic acid
—▽—	BPDA plus 4 mM vanillic acid
—▣—	BPDA plus 7 mM vanillic acid

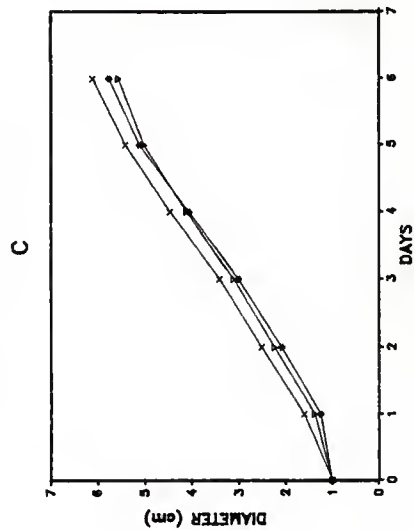
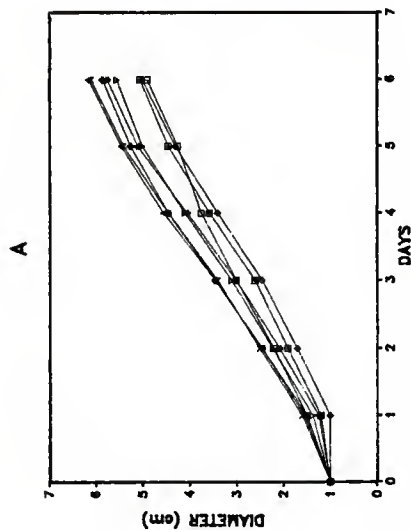
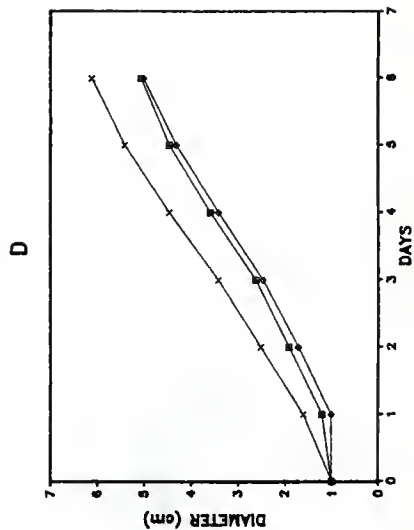
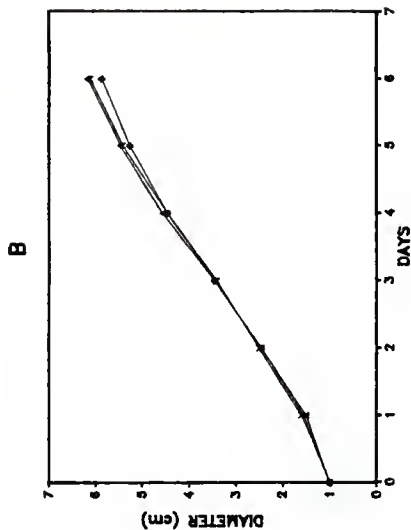


Fig. 64. Diameter of mycelial growth vs. days of culture for F. moniliforme against cinnamic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM cinnamic acid
—▽—	BPDA plus 4 mM cinnamic acid
—■—	BPDA plus 7 mM cinnamic acid

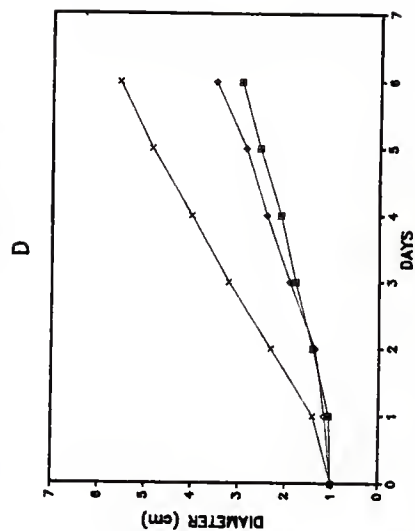
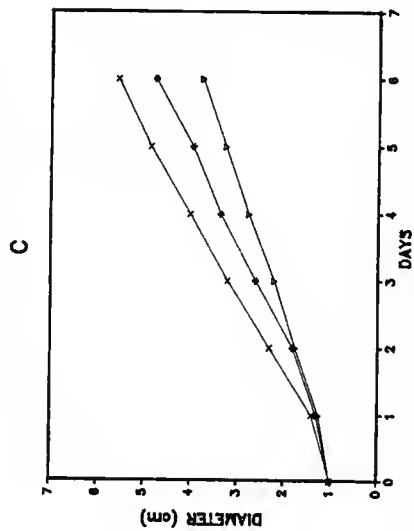
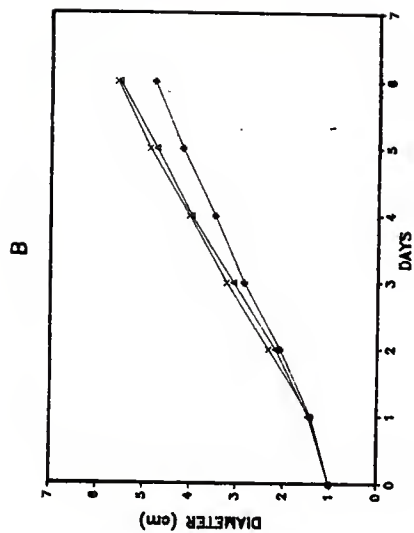
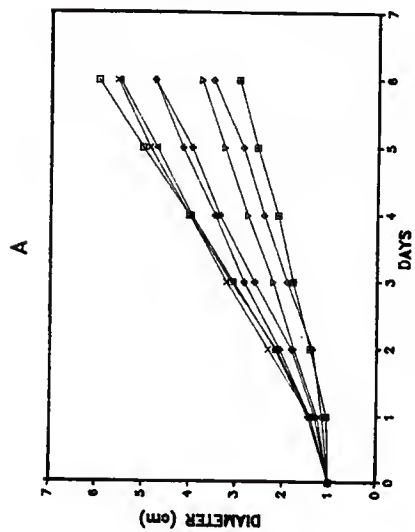
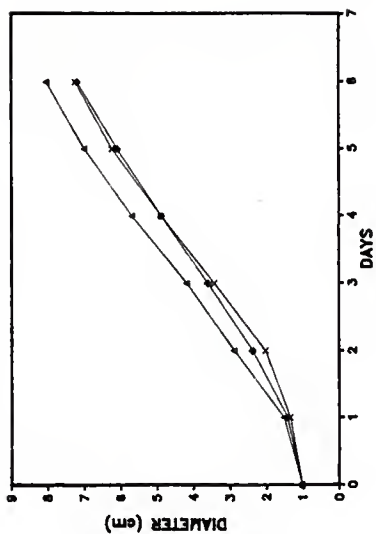


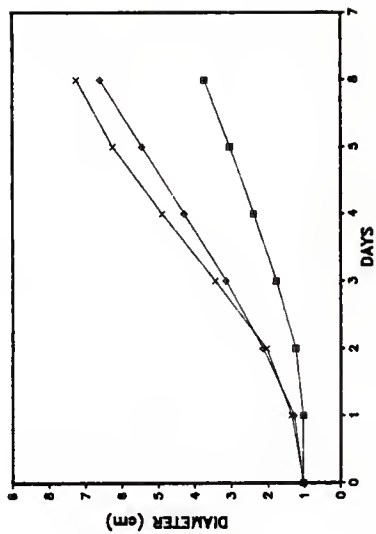
Fig. 65. Diameter of mycelial growth vs. days of culture for F. semitectum against cinnamic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM cinnamic acid
—▽—	BPDA plus 4 mM cinnamic acid
—⊠—	BPDA plus 7 mM cinnamic acid

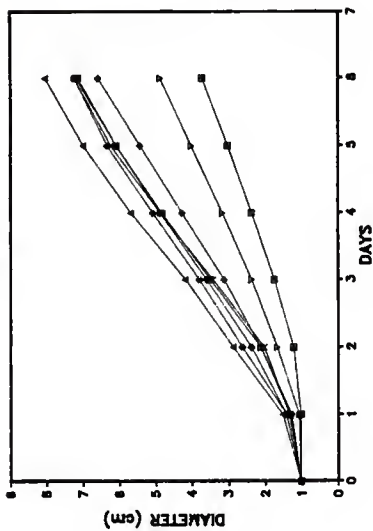
B



D



A



C

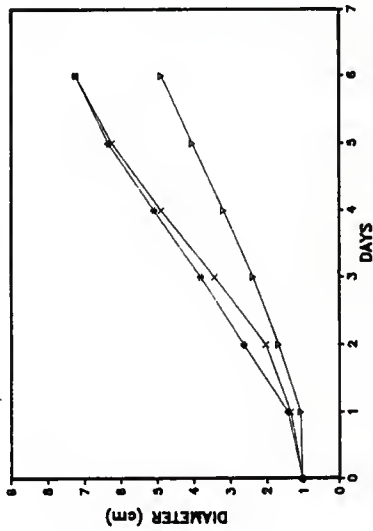
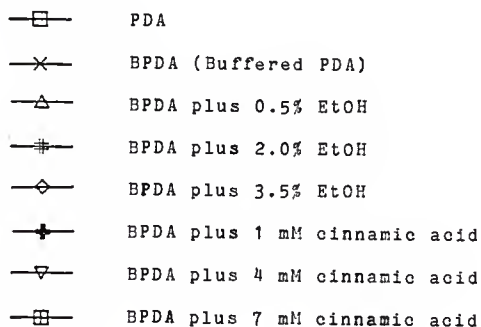


Fig. 66. Diameter of mycelial growth vs. days of culture for F. *equiseti* against cinnamic acid and controls.



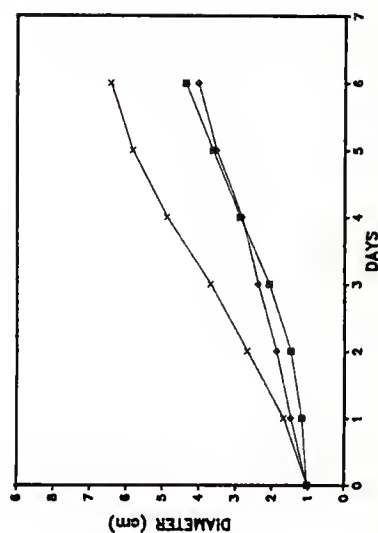
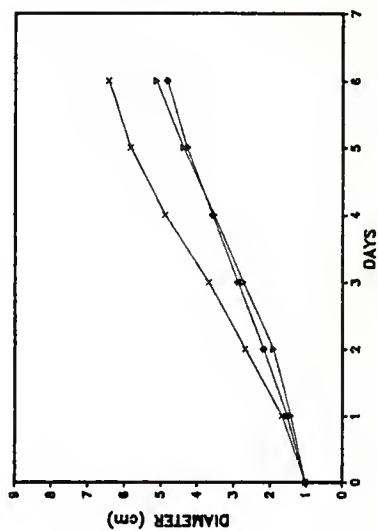
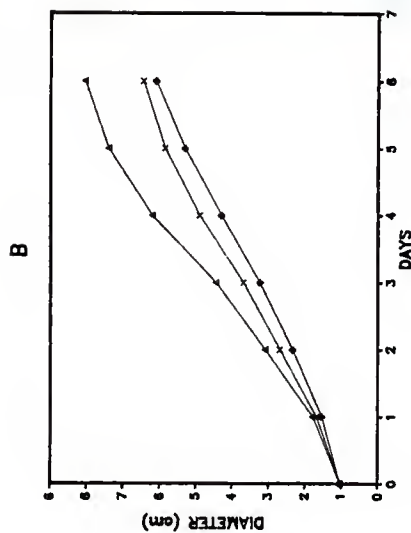
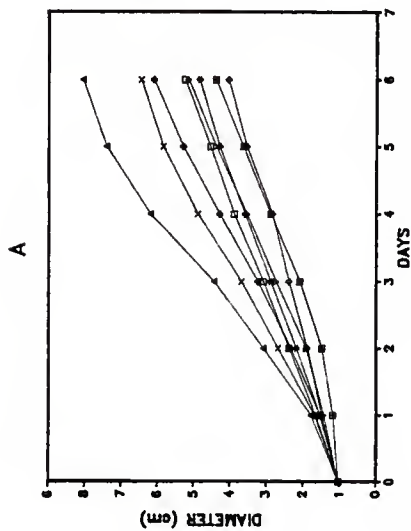
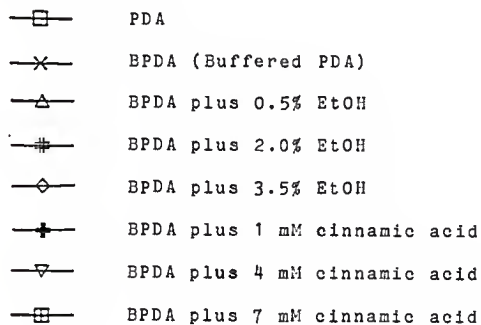
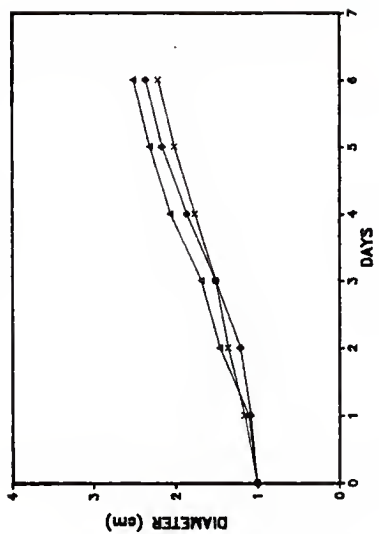


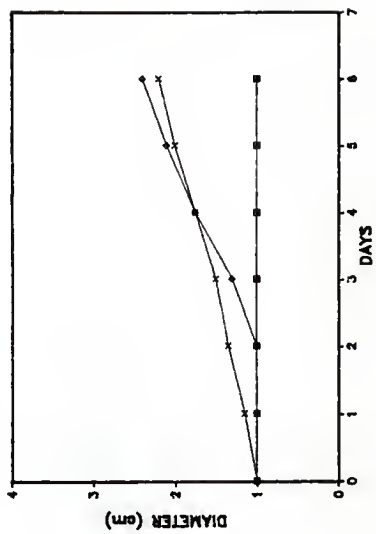
Fig. 67. Diameter of mycelial growth vs. days of culture for A. alternata against cinnamic acid and controls.



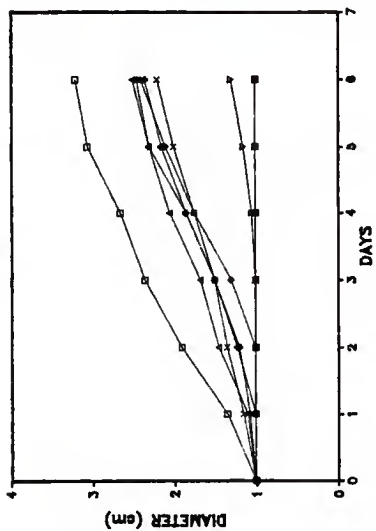
B



D



A



C

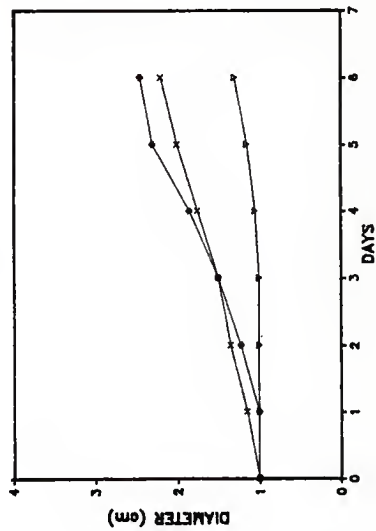
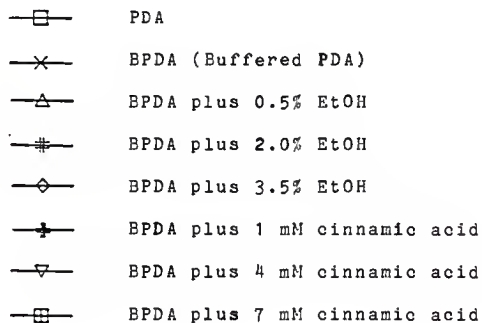
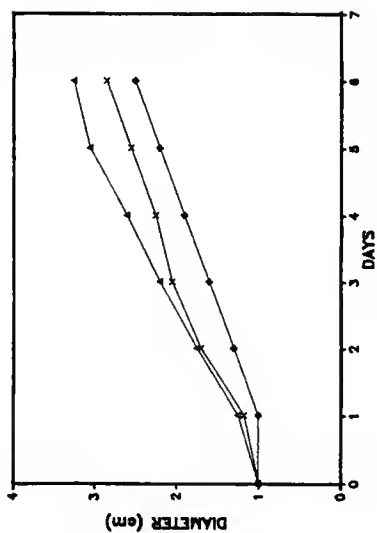


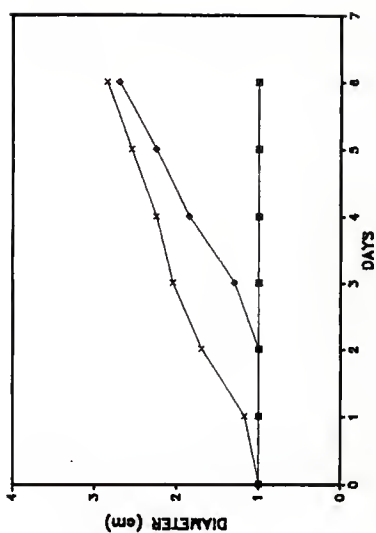
Fig. 68. Diameter of mycelial growth vs. days of culture for A. tenuissima cpx. against cinnamic acid and controls.



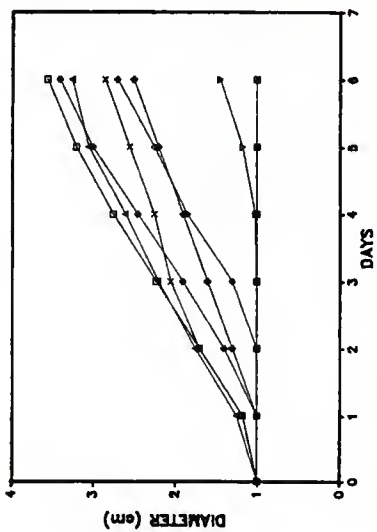
B



D



A



C

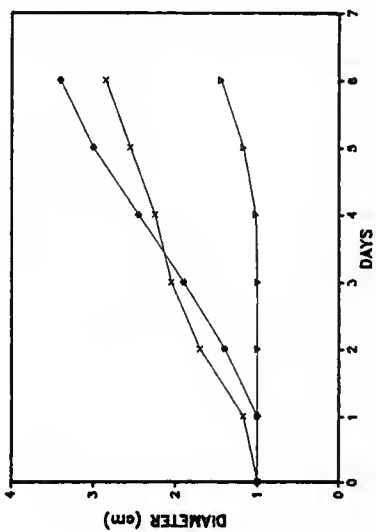
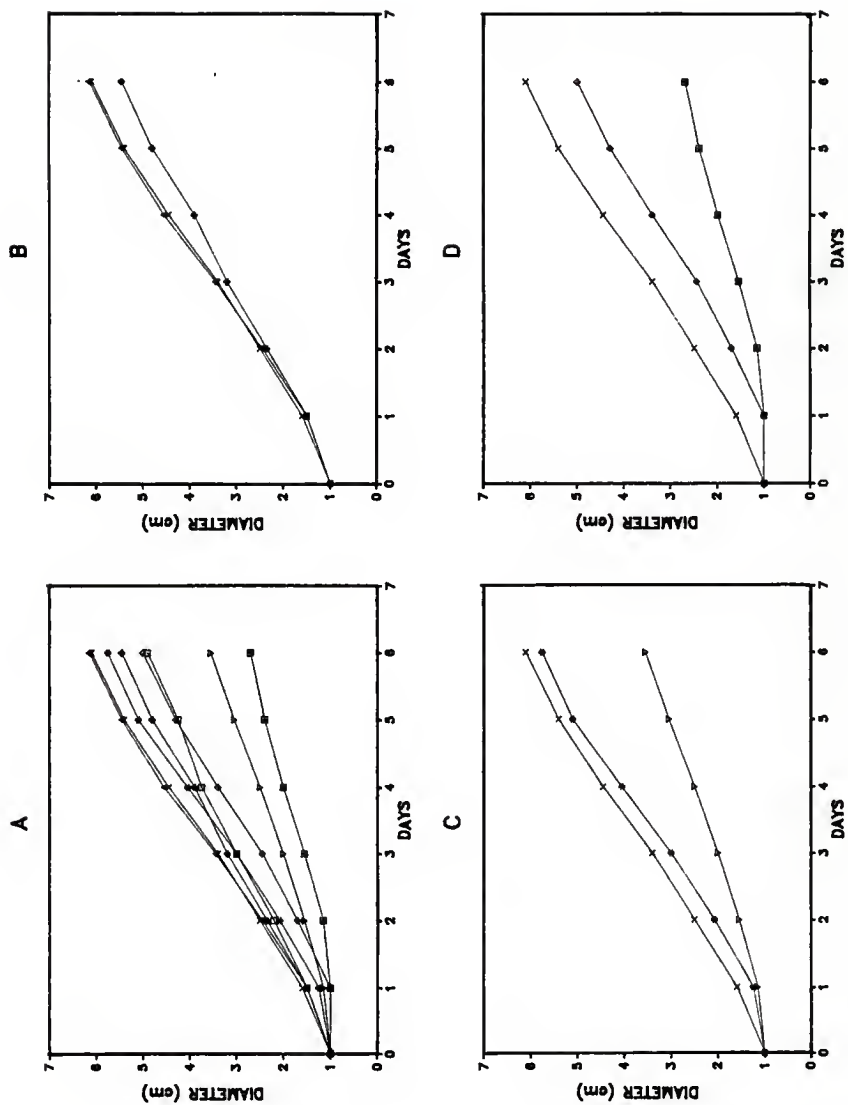


Fig. 69. Diameter of mycelial growth vs. days of culture for A. flavus against cinnamic acid and controls.

—□—	PDA
—X—	BPDA (Buffered PDA)
—△—	BPDA plus 0.5% EtOH
—#—	BPDA plus 2.0% EtOH
—◇—	BPDA plus 3.5% EtOH
—+—	BPDA plus 1 mM cinnamic acid
—▽—	BPDA plus 4 mM cinnamic acid
—■—	BPDA plus 7 mM cinnamic acid



### Polyphenols in sorghum

The Prussian blue (PB) test measures total polyphenols (Earp et al 1981, Hahn et al 1984, and Price and Butler 1977). It is based on the reduction by polyphenols (phenolic hydroxyl group) of ferric ion to ferrous ion, which in turn reacted with  $K_3Fe(CN)_6$  to form a ferricyanide-ferrous ion complex known as Prussian blue (Price and Butler 1977). The  $FeCl_3$  was prepared in acid medium (HCl) so that the Prussian blue complex formed slowly enough to be measured. The higher the polyphenol content, the darker the color.

The vanillin test measures proanthocyanidins (tannins) and leucoanthocyanidins (catechins) as well as with other polyphenolic compounds such as dihydrochalcones and flavanones (Earp et al 1981, Hahn et al 1984, and Sarkar and Howarth 1976). The A-ring and a single bond between  $C_2$  and  $C_3$  (Fig. 1C) are required for a positive vanillin reaction. Tannins are condensation products of flavan-3-ols and flavan-3,4-diols. Thus, they give positive reaction with vanillin (Gupta and Haslam 1980, and Sarkar and Howarth 1976).

The total polyphenols of sorghum samples by the PB, the vanillin hydrochloric acid (V-HCl), the modified vanillin hydrochloric acid (MV-HCl), and the MV-HCl (24-hr) methods are presented in Table 4. As expected for all four methods, SC0719, with a red pericarp, testa layer, and dominant spreader had a much higher polyphenol content than CS3541, which has a white pericarp with no pigmented testa. Since most of polyphenols (particularly tannins) are located in the testa layer, a sorghum variety with a pigmented testa is expected to have high amount of total polyphenols. Table 5 showed the same results for both of the free and bound extracts of both sorghum varieties by the PB and vanillin test. Further-

Table 4  
Total Polyphenols of Sorghum Samples

Sample	Methods that measure Catechin Equivalents (mg catechin per g sorghum)			
	PB	V-HCl	MV-HCl	MV-HCl(24 hr)
CS3541	0.64	23.27	38.50	69.75
SC0719	1.14	119.61	230.21	408.31

PB = Prussian Blue.

V-HCl = vanillin hydrochloric acid.

MV-HCl = modified vanillin hydrochloric acid.

Table 5

Total Polyphenols in Free and Bound Extracts of Sorghum Samples

Sample	Total Polyphenols (mg. catechin/g sorghum)			
	Free		Bound	
	PB	Vanillin	PB	Vanillin
CS3541	0.183	0.148	1.314	2.640
SC0719	10.725	3.130	17.438	4.433

PB = Prussian Blue

more, the CS3541 bound extract had more polyphenols than the free extract. The amount of polyphenols in the free and bound extracts are about the same in SC0719, but the bound form contains somewhat more than the free form.

#### HPLC separation

The free and bound extracts of sorghums CS3541 and SC0719 were also used for separation of substituted benzoic and cinnamic acids by using high performance liquid chromatography (HPLC). HPLC has been employed successfully for rapid and accurate qualitative and quantitative analysis of phenolic acids (Hagerman and Nicholson 1982, Hahn et al 1983, and Murphy and Stutte 1978). The chromatographic separation of the eight standards is illustrated in Figure 70. The analysis time was approximately 62 min., at a flow rate of 0.5 ml/min. Baseline resolution was achieved with all compounds. No appreciable baseline shift occurred due to absorbance by solvents.

The retention data with coefficients of variation (CV), capacity factors ( $k'$ ), and separation factors ( $\alpha$ ) achieved by the separation are shown in Table 6. Reproducibility of the separation was good. The CV of eight peaks was 1.17 or less. The CV values agreed with Hahn et al (1983) who found that the CV decreased as retention time ( $t_r$ ) increased because of the sharper peaks obtained by the use of gradient elution (increase in solvent strength over time). The reproducibility of peak areas (Table 7) was not as good as that of retention time, but was acceptable. The CV for most of the peak areas was less than 10% (3.23-9.46%). Only two of twenty four values were over 10%, one was near 10% (10.21%), and the other

Fig. 70. The chromatographic separation of the phenolic acid standards.

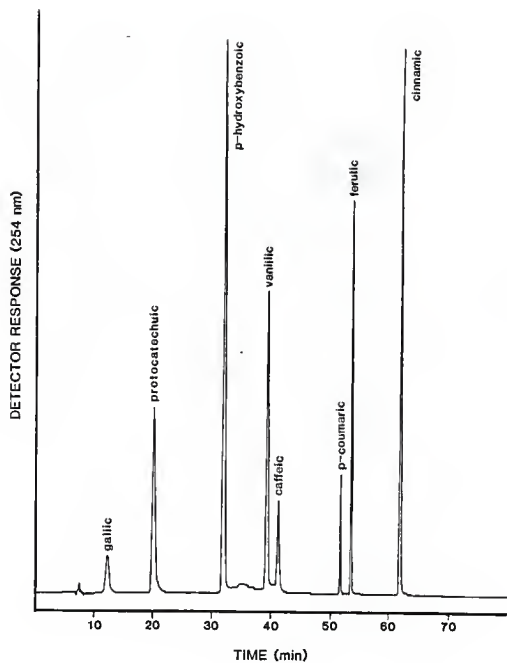


Table 6  
Chromatographic Data - I

Acid Compound	$t_r$ (min.)	CV (%)	$k'$	$\alpha$
Gallic	12.13 <sup>b</sup>	0.88	0.95	2.42
Protocatechuic	20.51	1.17	2.29	1.83
p-Hydroxybenzoic	32.37	0.93	4.20	1.28
vanillic	39.76	0.62	5.38	1.07
Caffeic	42.08	0.78	5.75	1.27
p-Coumaric	51.79	0.28	7.31	1.03
Ferulic	53.18	0.29	7.54	1.18
Cinnamic	61.75	0.31	8.91	

a => Flow rate of 0.5 ml/min.;  $t_o$  = 6.23 min.

b => Average of twelve runs.

Table 7  
Chromatographic Data - II<sup>a</sup>

Acid Compound	0.25ug/10ul		0.5ug/10ul		1.0ug/10ul		Corr. (r <sup>2</sup> )
	Area	CV(%)	Area	CV(%)	Area	CV(%)	
Gallic	4.32 <sup>b</sup>	7.25	8.12	7.94	16.56	4.28	0.9996
Protocatechuic	12.22	5.09	25.24	4.49	52.00	5.45	0.9996
p-Hydroxybenzoic	25.66	7.75	53.04	4.81	89.04	15.79	0.9868
Vanillic	13.24	4.32	26.52	7.73	56.80	6.64	0.9986
Caffeic	4.74	4.44	9.40	9.46	18.72	9.31	1.0000
p-Coumaric	3.40	4.90	6.64	4.61	12.80	10.21	0.9995
Ferulic	8.46	5.09	17.00	7.39	35.20	5.80	0.9996
Cinnamic	17.42	7.05	35.20	6.44	70.16	3.23	1.0000

a => measured by Planimeter.

b => Average of four runs.

had a CV of 15.79%. This resulted from detector noise and a slightly over range peak. This also affected the correlation value. The rest of the correlation values were good. In a multicomponent system,  $k'$  value should cluster around the optimum range, i.e.,  $1 \leq k' \leq 10$  (Murphy and Stutte 1978). All of the  $k'$  values fall within the optimum range ( $0.95 \leq k' \leq 8.91$ ), which are better than those reported by Hahn et al (1983), and Murphy and Stutte (1978).

Eight Phenolic acids were identified in sorghum extracts. In order of decreasing polarity they were: gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, ferulic, and cinnamic acids. Elution was in order of decreasing polarity, typical of reverse-phase chromatography (Johnson and Stevenson 1978). Fig. 71 illustrates the standard curves of eight phenolic acids. Hahn et al (1983) and Murphy and Stutte (1978) showed the relationship between the structure of the benzoic and cinnamic acids and their order of elution.

#### Extraction efficiency

The percent recovery of each phenolic acid standard using both of the free and bound acid extraction procedures were calculated (see Table 8). Extraction of free phenolic acids resulted in recoveries of 87.18-98.24% with CVs of 4.16-8.41%. The recoveries for the extraction of free phenolic acids were good. However, the recoveries for bound phenolic acids were much lower than those from the extraction of free phenolic acids, yielding 6.68-61.63% with CVs of 4.02-20.47%. This may be because the acids are destroyed or altered during acid hydrolysis (Hagerman and Nicholson 1982, and Krygier et al 1982). An additional factor in poor recoveries may

Fig. 71. Phenolic acid standard curves.

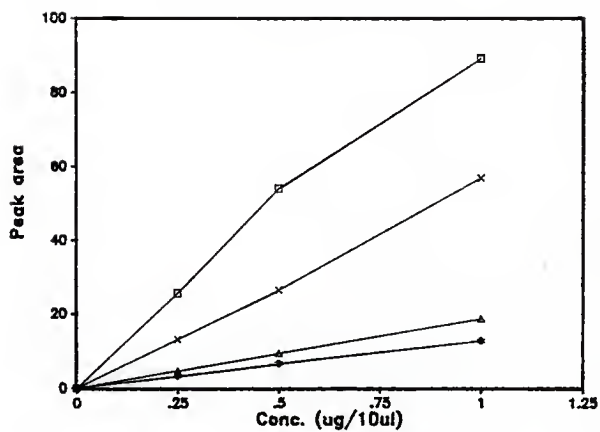
A

—□—	Ferulic acid
—X—	Cinnamic acid
—△—	Gallic acid
—#—	Protocatechuic acid

B

—□—	p-Hydroxybenzoic acid
—X—	Vanillic acid
—△—	Caffeic acid
—#—	p-Coumaric acid

A



B

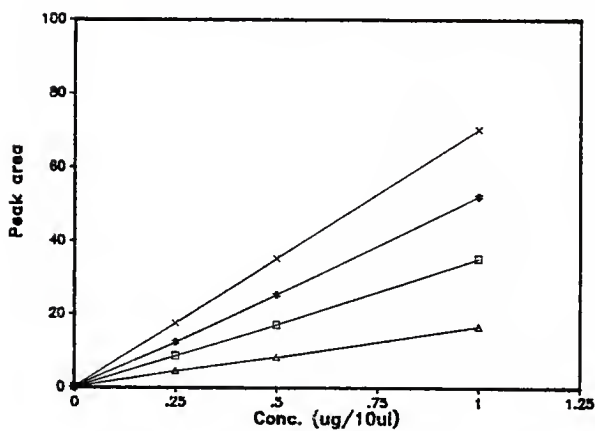


Table 8  
Extraction Recovery of Standard Phenolic Acids

Acid Compound	Free Extraction <sup>a</sup> Procedure		Bound Extraction <sup>b</sup> Procedure	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Gallic	92.64	4.16	51.06	9.08
Protocatechuic	91.54	8.41	57.52	10.31
p-Hydroxybenzoic	90.00	7.64	61.63	9.07
Vanillic	91.37	5.03	59.68	11.24
Caffeic	90.88	7.10	6.68	4.02
p-Coumaric	98.24	4.23	10.01	20.47
Ferulic	92.86	8.41	12.30	15.55
Cinnamic	87.18	6.87	59.23	9.63

a => Average of eight values.

b => Average of twelve values.

be the nature of the initial extraction system. A flocculant precipitate that is not soluble in either phase is present at the interface of ethyl acetate and water system. This makes it difficult to partition cleanly (Hahn et al 1983) and, this may reduce recoveries. The recoveries of caffeic, p-coumaric, and ferulic acids were much lower than the other standard compounds. This is the result of the conversion of their isomers during the bound acid extraction in addition to the effect of acid hydrolysis. This also affected the reproducibility (high CV values). Thus, accurate determination of these compounds after acid hydrolysis is probably not possible, although it is sometimes attempted (Charpentier and Cowles 1981, Hardin and Stutte 1980, and Murphy and Stutte 1978).

#### Phenolic acids in sorghum

The amounts of eight phenolic acids in the two sorghum samples are shown in Table 9. The majority of phenolic acids are present as bound acids. No protocatechuic acid was found among bound acids. All of the eight phenolic acids were found in CS3541 free extract. Very small amounts of gallic, protocatechuic, and cinnamic acids were present. All but gallic and protocatechuic acids were found in SC0719 free extract, of which caffeic and p-coumaric acids are the major species. Small peaks corresponding to gallic, protocatechuic, and cinnamic acids in the free form occurred infrequently and irreproducibly. This may result from the low solubility of the compound and its tendency to adsorb to glass (Billette et al 1981). Therefore, the unidentified phenolic acids (i.e. gallic and protocatechuic acids), particularly for the bound extracts may present in the small amount in the samples but not seen due to low

Table 9

Free and Bound Phenolic Acid Composition of Sorghum Samples

Acid Compound	Sorghum Samples ( $\mu\text{g/g}$ , dry weight basis)			
	CS3541		SC0719	
	Free	Bound	Free	Bound
Gallic	0.30	31.83	-	41.95
Protocatechuic	0.08	-	-	-
p-Hydroxybenzoic	5.44	10.14	2.78	10.22
Vanillic	2.30	268.10	2.19	193.53
Caffeic	6.16	224.55	20.36	471.56
p-coumaric	3.21	124.88	14.25	125.87
Ferulic	2.21	1,341.41	3.77	819.51
Cinnamic	0.08	75.98	0.02	39.00

recovery from the extracts.

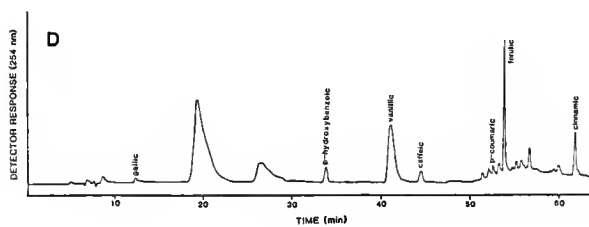
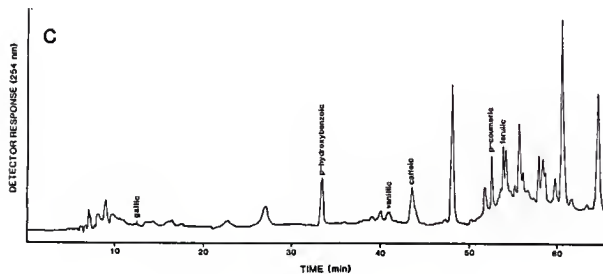
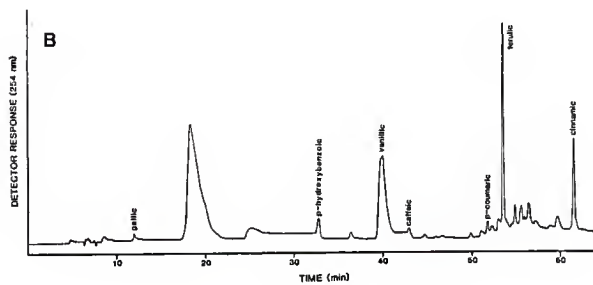
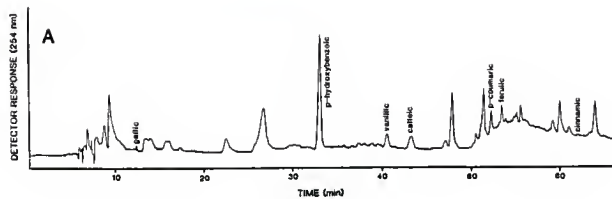
In addition to the above phenolic acids, there were several additional peaks present in the grain extract that were not identified (Fig. 72). There are several possible explanations for this. Either sorghum extract contained more than eight phenolic acids for which we had standards or not only benzoic and cinnamic acids were eluted with MeOH after applying the extract to the C-18 Sep-Pak cartridges. In the latter situation, other low M.W. phenolic compounds that pass through the cartridges may have been eluted along with the phenolic acids. In the bound extract, some peaks were almost certainly the isomers of the standard phenolic acids, i.e., caffeic, p-coumaric, ferulic, and cinnamic acids, which were converted during extraction procedure. In summary, the data showed that there is difference between the free and bound extracts of the same variety, but little difference in the distribution and amounts of those eight compounds in either the free or bound form.

#### Phenolic acids and resistance to fungal attack

Sorghum CS3541 and SC0719 are rated resistant to molding and weathering (Table 1). The results of the *in vitro* analysis for activity of sorghum extracts of both varieties against fungal growth indicated that the compounds effective against fungi were originally in the bound form. The bound extracts of both samples contain high amounts of phenolic acids (Table 9), more of which is present in CS3541 than in SC0719. However, the total polyphenol content of CS3541 is less than SC0719 (Table 5). As previously mentioned, the CS3541 bound extract is more effective than SC0719 bound extract in inhibiting fungal growth. Therefore, the total

Fig. 72. Free and bound phenolic acid chromatograms of sorghum CS3541 and SC0719.

- A. Free phenolic acids of CS3541
- B. Bound phenolic acids of CS3541
- C. Free phenolic acids of SC0719
- D. Bound phenolic acids of SC0719



polyphenol content does not reflect the resistance in sorghum. Tannins may or may not inhibit the fungal growth because SC0719 free extract, with high tannins, does not show the fungal growth inhibition. However, a slight inhibition was also observed (as previously discussed). Furthermore, CS3541 is relatively low in tannins but its bound extract is very effective against fungal growth. The resistant compounds therefore are likely to be non-tannin polyphenols (probably phenolic acids). In addition, phenolic acids found in high concentration in the bound extract (i.e. p-coumaric, ferulic, vanillic, and cinnamic acids), were shown to have inhibitory effects against fungal growth as the "pure" acid. Hahn et al (1983) found that sorghum grain resistant to fungal attack contained both a greater variety and larger amounts of the identified phenolic acids and unknown compounds than did susceptible varieties.

An alternative explanation for the activity of bound extracts centers on the changes induced by hydrolysis and extraction. Krygier et al (1982a, b) have demonstrated that acid hydrolysis results in uncontrolled oxidation of phenolic acids. As discussed in the literature review, oxidation of some phenolic compounds can increase their activity against fungal growth. Thus, it is possible that the growth inhibiting activity shown by bound extracts is due to the creation of highly potent oxidation products during acid hydrolysis. This possibility can be tested (i.e. by hydrolyzing and testing free extracts or model compounds).

Phenolic acids do not necessarily account for all the inhibitory activity against fungi. Tannins are reported to be important factors in resistance to fungi (Bullard and Elias 1980, and Gupta

and Haslam 1980). It might be true for some sorghum varieties but it is not in the case of sorghum CS3541 and probably not for SC0719. Tannins might be in part playing a role in resistance in addition to that played by phenolic acids. The inhibitory effect of tannins in the SC0719 free extract may be overshadowed by the stimulatory effects of other compounds of the extracts. Furthermore, other resistant compounds (i.e. flavonoids, unidentified compounds) may also be responsible for the resistance.

Different varieties of sorghums may have different resistance mechanisms. Sorghums resistant to one genus of fungus are not necessarily resistant to other genera due to differences in modes of fungal attack. Hahn et al (1983) suggests that the resistance mechanisms in sorghum are specific to the genus of fungi and/or mode of fungal attack. The mechanism associated with the presence of phenolic acids is, as yet, unexplained. However, their acidity (low pH) and toxicity may be the major reason for growth fungal inhibition. Their toxicity has been reported in other plants (Kosuge 1969, and Fridbam 1960). The astringency of tannins was reported to impart a degree of mold resistance or tolerance (Hahn et al 1984). The ability of tannins to bind and precipitate proteins (Butler et al 1980) and inhibit enzyme activity may give them antifungal properties (Hahn et al 1983).

The fungal growth stimulation of free extracts is an interesting observation. A similar observation was previously reported in other plant systems (Farkas and Kiraly 1962, Friend 1977, and Van Sumere 1960). The stimulatory effects may be due to the unidentified polyphenols present in free extracts. These polyphenols may provide carbon sources for the fungal growth. Westlake et al

(1961) reported that some fungi, including A. Flavus, Fusarium, and Alternaria, can utilize flavonoids and release carbon monoxide (an important, active, 1-carbon metabolite in the carbon cycle). However, unidentified stimulators of mycelial growth and spore germination changed to inhibitors after oxidation with either phenolase or peroxidase and  $H_2O_2$  in red clover plant (Friend 1977). The inhibitory action of the oxidized phenolic compounds was mediated through an inhibition of the action of the unidentified stimulator of spore germination and mycelial growth. This is consistent with observations made on the bound extracts. Therefore, the oxidized unidentified polyphenols in the free form may, in part, inhibit the fungal growth. This agrees well with tests on sorghum SC0719, which is rated very highly resistant due to its high content of unidentified polyphenols in addition to phenolic acids and tannins. Resistance to fungal attack could not be accounted solely to phenolic content or profile. Tannin content or unidentified polyphenols also contribute to inhibition of fungal growth.

## CONCLUSION

The plug outgrowth method for assessing the in vitro activity of non-tannin polyphenols against fungal growth was both accurate and repeatable. It was capable of quantitating the mycelial growth of all the fungi used in this study and demonstrating differences in their growth curves.

All model or purified individual phenolic acids of p-coumaric, ferulic, vanillic, and cinnamic acids were effective in inhibiting fungal growth in vitro. The inhibition was affected by both the acids and the species of fungus. In order of decreasing antifungal activity, the acids were; cinnamic > ferulic > p-coumaric > vanillic acid. Decreasing the polarity of phenolic acid increased its inhibitory activity. Cinnamic acid inhibited all fungal growth, even at concentration as low as 1 mM. The growth inhibition by ferulic acid started at 1 mM for most of the fungi tested, except for A. flavus which occurred at 4 mM. p-Coumaric acid inhibited F. equiseti, F. semitectum, and A. tenuissima cpx. at 1 mM, F. moniliforme at 4 mM, and A. alternata at 7 mM. No inhibition was observed at any concentration tested for A. flavus. Vanillic acid, which was the least effective of the four phenolic acids, inhibited F. equiseti at 1 mM, F. semitectum at 7 mM. Slight inhibition was observed at 1 mM for F. moniliforme. There was no inhibition detected for Alternaria spp. and A. flavus. In order of decreasing sensitivity to phenolic acids, fungi were ranked as; F. equiseti > F. moniliforme > F. semitectum > A. tenuissima cpx. > A. alternata > A. flavus.

When phenolic acid extracts were tested, the inhibition of

fungus growth was apparently due to the acids originally in the bound form. These extracts of both samples showed similar inhibitory effects on fungus growth as did the model phenolic acids. They affected Fusarium > Alternaria > A. flavus. The growth inhibition by the CS3541 bound extract was observed at 0.16, 0.37, and 0.79 mg catechin equivalent/milliliter (CE/ml) for Fusarium, Alternaria, and A. flavus respectively. SC0719 bound extract inhibited Fusarium at 2.33 mg CE/ml but Alternaria and A. flavus at 23.3 mg CE/ml. Complete inhibition occurred at 23.3 mg CE/ml for all fungi except A. flavus. However, the free phenolic acid extracts stimulated fungus growth. Oxidation of the bound acids (during hydrolysis) to potent inhibitors of fungus growth is a possible explanation for the difference between free and bound extracts.

Eight phenolic acids in the sorghum extracts were identified by using HPLC: gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, ferulic, and cinnamic. The majority of these acids were present in the "bound" form. All of the eight phenolic acids (except protocatechuic acid) were present as bound form in both samples. However, low concentrations of p-hydroxybenzoic, vanillic, p-coumaric, ferulic, and cinnamic acids were present in the free acid extract of SC0719. All of the eight phenolic acids were found in CS3541 free acid extract. By comparing fungus growth inhibition between purified phenolic acids and the polyphenol enriched extract, it was concluded that phenolic acids originally in the bound form were active in the growth inhibition seen for both sorghums. However, resistance to fungus attack could not be accounted solely to phenolic content or profile. Tannin content or unidentified polyphenols or changes due to extraction conditions

may also contribute to fungal resistance and the observed in vitro results.

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## APPENDIX

TABLE A  
Mycelial Growth (cm) of Fungi Grown on PDA with Varying Concentrations of  
CS3541 Free Extract (in 80% EtOH) and 80% EtOH (as control)

Fungi	PDA	CS3541 Free Extract (mg catechin equi. per ml PDA)					80% EtOH (%) <sup>a</sup>	
		0.07	0.29	1.02	0.25	1.0	3.5	
<u>F. moniliforme</u>	<sup>b</sup>	8.50±0.00	8.45±0.06	8.50±0.00	8.50±0.00	7.95±0.06	7.90±0.12	6.65±0.06
<u>F. semitectum</u>		8.50±0.00	8.50±0.00	8.50±0.00	8.50±0.00	8.50±0.00	8.50±0.00	7.85±0.18
<u>F. equiseti</u>		8.05±0.06	8.50±0.00	8.50±0.00	8.50±0.00	7.65±0.42	6.60±0.00	7.20±0.00
<u>A. alternata</u>		4.65±0.18	3.88±0.21	4.15±0.18	6.13±0.15	4.45±0.06	4.45±0.18	4.80±0.00
<u>A. tenuissima</u> cpx.		5.35±0.06	4.10±0.12	4.30±0.00	5.75±0.30	5.40±0.00	5.35±0.06	5.60±0.12
<u>A. flavus</u>		5.98±0.03	6.35±0.06	6.90±0.00	7.55±0.06	6.20±0.00	6.20±0.00	6.10±0.12

a => 80% EtOH at 0.25, 1.0, 3.5% concentration are controls for CS3541 Free Extract at 0.07, 0.29, 1.02 mg catechin equivalent per ml PDA, respectively.

b => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE B  
Mycelial Growth (cm) of Fungi Grown on PDA with Varying Concentrations of  
CS3541 Bound Extract (in 80% EtOH) and 80% EtOH (as control)

Fungi	CS3541 Bound Extract				80% EtOH (%) <sup>a</sup>			
	PDA	(mg catechin equi. per ml PDA)						
		0.18	0.72	1.80	0.50	2.0	5.0 <sup>b</sup>	
<i>E. moniliforme</i>		8.50±0.00 <sup>c</sup>	4.80±0.12	1.00±0.00	1.00±0.00	7.55±0.06	6.90±0.00	3.50±0.00
<i>E. semitectum</i>		8.50±0.00	4.75±0.30	1.00±0.00	1.00±0.00	8.50±0.00	8.15±0.06	2.70±0.00
<i>E. equiseti</i>		8.05±0.06	5.65±0.06	1.00±0.00	1.00±0.00	6.55±0.30	6.30±0.00	3.40±0.12
<i>A. alternata</i>		4.65±0.18	3.80±0.00	1.00±0.00	1.00±0.00	4.55±0.06	4.55±0.18	2.00±0.00
<i>A. tenuissima</i> cpx.		5.35±0.06	3.25±0.06	1.00±0.00	1.00±0.00	5.40±0.00	5.70±0.00	1.75±0.06
<i>A. flavus</i>		5.98±0.03	6.00±0.00	2.00±0.00	1.00±0.00	6.20±0.12	6.35±0.06	5.50±0.00

a => 80% EtOH at 0.50, 2.0, 5.0% concentration are controls for CS3541 Bound Extract at 0.18, 0.72, 1.80 mg catechin equivalent per ml PDA, respectively.

b => 5.0% EtOH plus tartaric acid to control same pH as extract.

c => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE C

Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of SC0719 Free Extract (in 80% EtOH) and 80% EtOH (as control)

Fungi	SC0719 Free Extract (mg catechin equi. per ml PDA)				80% EtOH (%) <sup>b</sup>	
	PDA	1.65	6.60	16.5	0.5	2.0
<i>E. moniliforme</i>	7.50±0.12 <sup>c</sup>	7.00±0.00	7.00±0.12	6.50±0.00	7.30±0.00	6.85±0.18
<i>E. semitectum</i>	8.50±0.00	8.50±0.00	8.20±0.00	8.20±0.00	8.50±0.00	8.50±0.00
<i>E. equiseti</i>	7.95±0.30	8.50±0.00	8.50±0.00	8.50±0.00	7.15±0.18	6.75±2.12
<i>A. alternata</i>	2.95±0.06	3.48±0.03	3.70±0.00	3.85±0.06	3.00±0.12	3.25±0.06
<i>A. tenuissima</i> cpx.	4.20±0.00	3.88±0.09	4.10±0.00	3.90±0.00	4.10±0.12	3.95±0.06
<i>A. flavus</i>	7.60±0.00	7.95±0.06	7.90±0.00	7.95±0.06	6.95±0.18	6.65±0.06

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.5, 2.0, 5.0% concentration are controls for SC0719 Free Extract at 1.65, 6.60, 16.50 mg catechin equivalent per ml PDA, respectively.

c => Average from six measurements of fungal growth on day 6 ( $\pm$  95% confidence interval).

TABLE D  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
SC0719 Bound Extract (in 80% EtOH) and 80% EtOH (as control)

Fungi	SC0719 Bound Extract (mg catechin equi. per ml PDA)					80% EtOH (%) <sup>b</sup>	
	PDA	2.33	9.30	23.3	0.5	2.0	5.0 <sup>c</sup>
<hr/>							
<i>F. moniliforme</i>	7.50±0.12 <sup>d</sup>	6.68±0.03	5.30±0.12	1.00±0.00	7.30±0.00	6.85±0.18	3.68±0.03
<i>F. semitectum</i>	8.50±0.00	8.50±0.00	5.80±0.00	1.00±0.00	8.50±0.00	8.50±0.00	4.10±0.12
<i>F. equiseti</i>	7.95±0.30	8.50±0.00	6.70±0.12	1.00±0.00	7.15±0.18	6.75±2.12	2.80±0.24
<i>A. alternata</i>	2.95±0.06	3.50±0.12	3.50±0.00	1.00±0.00	3.00±0.12	3.25±0.06	3.50±0.36
<i>A. tenuissima</i> cpx.	4.20±0.00	4.15±0.06	4.10±0.12	1.00±0.00	4.10±0.12	3.95±0.06	3.65±0.06
<i>A. flavus</i>	7.60±0.00	7.60±0.24	6.30±0.00	3.80±0.00	6.95±0.18	6.65±0.06	6.90±0.48

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.5, 2.0, 5.0% concentration are controls for SC0719 Bound Extract at 2.33, 9.30,

23.3 mg catechin equivalent per ml PDA, respectively.

c => 5.0% EtOH plus tartaric acid to control same pH as extract.

d => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE E  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
CS3541 Free Extract (in 80% EtOH) and 80% EtOH (as control)

Fungi	CS3541 Free Extract (mg catechin equi. per ml PDA)					80% EtOH (%) <sup>b</sup>	
	PDA	0.02	0.11	0.22	0.3	1.5	3.0
<i>E. moniliforme</i> <sup>c</sup>	5.75±0.06	5.40±0.12	5.35±0.06	5.15±0.06	5.50±0.00	4.95±0.06	3.75±0.18
<i>E. semitectum</i>	8.00±0.00	7.95±0.06	7.55±0.18	7.65±0.06	8.00±0.00	7.95±0.06	6.90±0.12
<i>E. equiseti</i>	4.90±0.12	4.10±0.00	4.30±0.12	7.45±0.06	4.75±0.30	4.40±0.12	5.20±0.36
<i>A. alternata</i>	2.35±0.06	2.85±0.06	3.30±0.00	3.70±0.12	2.40±0.00	3.10±0.00	2.35±0.06
<i>A. tenuissima</i> cpx.	2.25±0.06	3.35±0.06	3.75±0.06	4.00±0.00	3.15±0.06	3.55±0.06	2.45±0.55
<i>A. flavus</i>	5.30±0.00	5.90±0.12	5.70±0.12	5.85±0.06	5.30±0.00	5.10±0.12	4.55±0.06

a -> Prepared with phosphate buffer, pH 6.4.

b -> 80% EtOH at 0.3, 1.5, 3.0% concentration are controls for CS3541 Free Extract at 0.02, 0.11, 0.22 mg catechin equivalent per ml PDA, respectively.

c -> Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE F  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
CS3541 Bound Extract (in 80% EtOH) and 80% EtOH (as control)

Fungi	CS3541 Bound Extract (mg catechin equi. per ml PDA)				b	
	PDA	0.16	0.37	0.79	0.3	0.7
						1.5 <sup>c</sup>
<i>E. moniliforme</i>	5.75±0.06	4.65±0.06	4.15±0.06	2.45±0.30	5.50±0.00	5.85±0.06
<i>F. semitectum</i>	8.00±0.00	6.50±0.24	5.60±0.12	1.00±0.00	8.00±0.00	7.90±0.12
<i>F. equiseti</i>	4.90±0.12	4.40±0.24	5.75±0.18	2.85±0.18	4.75±0.30	4.85±0.06
<i>A. alternata</i>	2.35±0.06	3.00±0.00	2.85±0.06	1.15±0.18	2.40±0.00	3.65±0.30
<i>A. tenuissima</i> cpx.	2.25±0.06	3.30±0.00	3.20±0.00	1.05±0.06	3.15±0.06	3.65±0.18
<i>A. flavus</i>	5.30±0.00	5.15±0.06	4.95±0.06	3.35±0.18	5.30±0.00	4.65±0.06

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.3, 0.7, 1.5% concentration are controls for CS3541 Free Extract at 0.16, 0.37, 0.79 mg catechin equivalent per ml PDA, respectively.

c => 1.5% EtOH plus tartaric acid to control same pH as extract.

d => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE G  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
p-Coumaric Acid (in 80% EtOH) and 80% EtOH (as control)

Fungi	PDA	p-Coumaric Acid (mM)				80% EtOH (%) <sup>b</sup>	
		1	4	7	0.5	2.0	3.5
<i>E. moniliforme</i>	5.55±0.06 <sup>c</sup>	5.30±0.00	3.90±0.12	3.25±0.06	5.50±0.12	4.75±0.06	3.50±0.12
<i>F. semitectum</i>	7.20±0.00	7.45±0.18	5.95±0.18	4.30±0.00	8.00±0.00	7.20±0.24	6.55±0.06
<i>F. equiseti</i>	6.40±1.94	4.35±0.06	4.15±0.18	3.80±0.24	8.00±0.00	4.80±0.24	4.00±0.24
<i>A. alternata</i>	2.20±0.00	2.40±0.12	2.40±0.12	1.65±0.42	2.50±0.00	2.45±0.06	2.40±0.12
<i>A. tenuissima</i> cpx.	2.85±0.06	2.80±0.00	2.25±0.18	2.00±0.12	3.25±0.30	3.40±0.00	2.70±0.24
<i>A. flavus</i>	6.10±0.00	6.05±0.06	5.65±0.06	4.95±0.18	6.15±0.06	5.75±0.18	5.00±0.00

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.5, 2.0, 3.5% concentration are controls for p-coumaric acid at 1, 4, 7 mM, respectively.

c => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE H  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
Ferulic Acid (in 80% EtOH) and 80% EtOH (as control)

Fungi	PDA	Ferulic Acid (mM)					80% EtOH (%) <sup>b</sup>		
		1	4	7	0.5	2.0	3.5		
<u>E. moniliforme</u>	<sup>c</sup>	5.55±0.06	4.75±0.06	3.75±0.06	2.95±0.06	5.50±0.12	4.75±0.06	3.50±0.12	
<u>E. semitectum</u>		7.20±0.00	6.70±0.12	4.20±0.00	3.75±0.06	8.00±0.00	7.20±0.24	6.55±0.06	
<u>E. equiseti</u>		6.40±1.94	4.60±0.24	4.65±0.06	4.55±0.30	8.00±0.00	4.80±0.24	4.00±0.24	
<u>A. alternata</u>		2.20±0.00	2.35±0.18	2.85±0.06	2.55±0.06	2.50±0.00	2.45±0.06	2.40±0.12	
<u>A. tenuissima</u> cpx.		2.85±0.06	2.95±0.06	2.65±0.06	2.25±0.06	3.25±0.30	3.40±0.00	2.70±0.24	
<u>A. flavus</u>		6.10±0.00	5.90±0.12	5.25±0.06	4.15±0.06	6.15±0.06	5.75±0.18	5.00±0.00	

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.5, 2.0, 3.5% concentration are controls for ferulic acid at 1, 4, 7 mM, respectively.

c => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE I  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
Vanillic Acid (in 80% EtOH) and 80% EtOH (as control)

Fungi	Vanillic Acid (mM)					80% EtOH (%) <sup>b</sup>	
	PDA					1	3.5
<u>F. moniliforme</u>	5.55±0.06	5.15±0.06	4.50±0.00	3.55±0.06	5.50±0.12	4.75±0.06	3.50±0.12
<u>F. semitectum</u>	7.20±0.00	8.00±0.00	7.25±0.06	5.05±0.30	8.00±0.00	7.20±0.24	6.55±0.06
<u>F. equiseti</u>	6.40±1.94	4.55±0.06	4.75±0.06	4.45±0.30	8.00±0.00	4.80±0.24	4.00±0.24
<u>A. alternata</u>	2.20±0.00	2.50±0.12	3.20±0.12	2.90±0.12	2.50±0.00	2.45±0.06	2.40±0.12
<u>A. tenuissima</u> cpx.	2.85±0.06	3.35±0.06	3.35±0.18	2.85±0.06	3.25±0.30	3.40±0.00	2.70±0.24
<u>A. flavus</u>	6.10±0.00	5.85±0.06	5.55±0.06	5.05±0.06	6.15±0.06	5.75±0.18	5.00±0.00

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.5, 2.0, 3.5% concentration are controls for vanillic acid at 1, 4, 7 mM, respectively.

c => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

TABLE J  
Mycelial Growth (cm) of Fungi Grown on buffered PDA<sup>a</sup> with Varying Concentrations of  
Cinnamic Acid (in 80% EtOH) and 80% EtOH (as control)

Fungi	Cinnamic Acid (mM)				80% EtOH (%) <sup>b</sup>	
	PDA	1	4	7	0.5	2.0
<i>P. moniliforme</i> <sup>c</sup>	5.55±0.06	4.75±0.06	3.65±0.06	2.75±0.18	5.50±0.12	4.75±0.06
<i>F. semitectum</i>	7.20±0.00	7.15±0.06	4.85±0.79	3.70±0.12	8.00±0.00	7.20±0.24
<i>F. equiseti</i>	6.40±1.94	6.05±1.15	5.10±0.00	4.35±0.18	8.00±0.00	4.80±0.24
<i>A. alternata</i>	2.20±0.00	2.35±0.06	1.30±0.12	1.00±0.00	2.50±0.00	2.45±0.06
<i>A. tenuissima</i> cpx.	2.85±0.06	2.50±0.00	1.45±0.06	1.00±0.00	3.25±0.30	3.40±0.00
<i>A. flavus</i>	6.10±0.00	5.45±0.06	3.55±0.06	2.70±0.12	6.15±0.06	5.75±0.18

a => Prepared with phosphate buffer, pH 6.4.

b => 80% EtOH at 0.5, 2.0, 3.5% concentration are controls for cinnamic acid at 1, 4, 7 mM, respectively.

c => Average from six measurements of fungal growth on day 6 (± 95% confidence interval).

IN VITRO ACTIVITY OF SORGHUM NON-TANNIN POLYPHENOLS  
ON GROWTH OF POTENTIAL MYCOTOXIN-PRODUCING FUNGI

by

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## ABSTRACT

Two grain mold and weathering resistant sorghum varieties that differed in tannin content were used for in vitro analysis of the effects of non-tannin polyphenols on the growth of potential myco-toxin-producing fungi. The fungi selected for testing included: Fusarium moniliforme, Fusarium semitectum, Fusarium equiseti, Alternaria alternata, Alternaria tenuissima cpx., and Aspergillus flavus. Fungal growth was determined in vitro on PDA or buffered PDA by a plug outgrowth method. Colony diameter on the control and experimental plates was measured daily for six days.

Four phenolic acids occurring in sorghum (ferulic, p-coumeric, vanillic, and cinnamic acids) were chosen as model compounds and tested in vitro at 1mM, 4mM, and 7mM. The extent of growth inhibition and minimum inhibitory concentration depended upon the fungus tested. In order of decreasing antifungal activity, the acids were ranked as cinnamic > ferulic > p-coumaric > vanillic acid. In addition, activity was affected by the polarity of the acids.

Free and bound phenolic acid extracts from sorghum CS3541 and SC0719 were used at increasing concentrations in a study of in vitro activity of polyphenols against fungal growth. Inhibition of fungal growth was observed in the presence of the bound acids. The free phenolic acid extracts stimulated fungal growth.

Using HPLC (high performance liquid chromatography), eight phenolic acids were identified in the sorghum extracts tested. They were; gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, ferulic, and cinnamic. The majority of phenolic acids are present in the "bound" form. However, low concentra-

tions of p-Hydroxybenzoic, vanillic, p-coumaric, ferulic, and cinnamic acids were found in the free form in extracts of SC0719. All of the eight phenolic acids were found in SC3541 free acid extract. By comparing fungal growth inhibition between purified phenolic acids and the polyphenol enriched extract, was concluded that phenolic acids in bound form were active in the observed growth inhibition. However, resistance to fungal attack could not be accounted solely to phenolic content or profile. Tannin content or unidentified polyphenols also contribute to inhibition of fungal growth.